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REFECTION IN THE RAT[1]

WITH AN APPENDIX ON METHODS OF PREPARING BASIC MATERIALS FOR DEFICIENT DIETS

SIDNEY BLISS

WITH THE TECHNICAL ASSISTANCE OF FRED GREEN

Department of Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana

EIGHT FIGURES

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HISTORICAL

Refection was first reported from the Copenhagen Laboratory of Fridericia in 1926, and was observed independently by Roscoe ('27).

Refection refers to a condition in experimental animals by virtue of which they are able to grow and reproduce, even in the second generation, on diets believed to be free of the undifferentiated vitamin B (Fridericia, '26).

If one is to accept the phenomenon of refection as showing what it purports to do, it must be assumed that refected animals are capable of vitamin B synthesis. Another feature of this condition is the presence of bulky, white feces in the rat. The bulk is due to occluded air, and the whiteness is due to the presence in the feces of undigested starch grains. It has been stated that these animals do not digest raw starch in normal fashion, even though the starch grains, once they have been separated from the white feces, can then be digested under appropriate conditions. Curiously enough, the feces from refected rats contain amylase, along with undigested starch.

[1] This investigation was aided by a grant from the David Trautman Schwartz Research Fund.

The subject has been well reviewed in Brownings' excellent monograph on the vitamins ('31).

The original claim of Fridericia that there is such a condition as refection has been confirmed at the Lister Institute in London by Roscoe ('27), in Hopkins Laboratory at Cambridge by Kon and Watchorn ('27), in the veterinarian-physiological laboratory at Leipzig by Scheunert and collaborators ('29), and at the Pasteur Institute of Burmah by Taylor and Thant ('29).

Two publications on refection have appeared in the American literature. Mendel and Vickery ('29) dismissed the subject in a short negative report. From their paper one gains the impression that they felt that those who claim refection in animals may have been working with diets that were less deficient in vitamin B than supposed. After our work had been in progress for a year, a second American publication confirmed the European work. In a very short statement, Parsons, Kelley and Hussemann ('33) state that "Bulky white feces containing numerous starch grains, similar to feces reported by others as typical of refection, occurred spontaneously in individual rats on a vitamin G-low ration containing raw potato starch, etc." This is the only work from this country that we have been able to interpret as confirmation of the work of Fridericia.

The one unsuccessful attempt at obtaining refection was that of Mendel and Vickery ('29), and so far as we know these last two papers represent the only contributions to the subject of refection from the United States.

DEFINITION

Because refection has been adequately reviewed (Browning, '31) with the appearance of but few papers since Browning's monograph, we will not attempt a complete discussion of the subject. Briefly, refection in the rat carries with it the idea that vitamin B is synthesized, most probably in the lower gut. Co-existing in the feces of refected rats are considerable quantities of undigested raw starch as well as amylase which can be shown to be active on fresh raw starch. The starch

which has been separated from refected feces can be digested by starch-splitting enzymes if it is given a preliminary treatment with acid alcohol, this extraction presumably removing something which is there as a coating on the grains, and which in the rat had prevented the digestion of the starch in the first place. One rat may refect another by contagion, and, whatever the refectious agent may be, it appears to be destroyed when subjected to rigid heat sterilization. Vitamin B synthesis and sub-normal starch digestion seem to go hand-in-hand; neither one appears separately.

It is reported that refection can be obtained only with raw starch in the diet (Roscoe, '27). Cooked starch or other soluble carbohydrate will not allow of refection. The ease and certainty of obtaining refection varies with the kind of raw starch fed. Refection is more difficult with corn starch, whereas it frequently occurs with rice starch, and seems most certain to occur with potato starch.

ATTEMPTS AT CONFIRMATION

We began our experiments with the idea of confirming or disproving refection. Our first effort was to attempt to obtain refection. After we had been successful in this respect our chief aim was to find out whether or not the successful growth of rats on diets containing raw potato starch, and very low in vitamin B, could be explained on the basis of unaccounted-for vitamin-B contamination of the starch.

EXPERIMENTAL

A. An attempt to confirm the work of Fridericia on refection

Experiment 1. The diets were copied closely from those used by Fridericia in order to determine whether or not we could confirm his results.

Our control rat received the following diet:

	Per cent
Casein	20
Sucrose	52
Butter fat	15
Agar	3
Salt[1]	5
Yeast	5

[1] McCollum Davis salt mixture.

The control rat I grew normally, see figure no. 1. Rat V received this diet without the yeast supplement—and the extra 5 per cent thus available to the diet was added to the casein portion, making the percentage of casein 25 instead of 20.

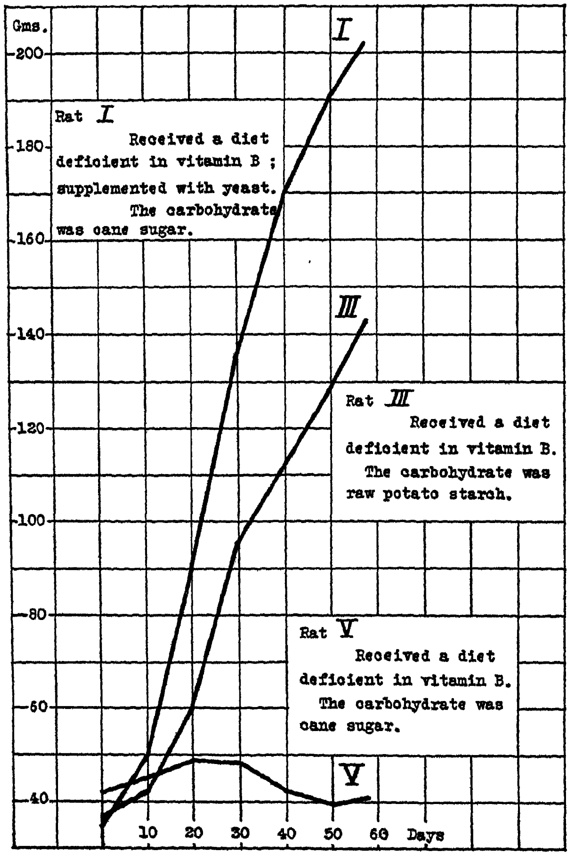

Figure 1

This rat, V, declined in weight, and died with typical symptoms of vitamin-B deficiency (fig. 1). Rat III of this series received the same diet as the other deficient rat (V) except that in the case of the carbohydrate, raw potato starch was substituted for cane sugar. The subsequent history of this

animal is essentially that of a normal animal. It grew so well and appeared in such excellent condition that it was indistinguishable from the normal control (I) receiving 0.5 gm. of dried yeast daily.[2] Examination of the growth curves leaves no doubt that, after an initial period of lag during which refection becomes established, there was normal growth in rat III in addition to general well being, even though it received a diet as devoid of vitamin B as the other rat (V) which failed to grow, with the sole exception of whatever difference, if any, there may have been between the vitamin-B content of raw potato starch and sucrose.

In addition to the favorable weight response, we also had the other criteria of refection—namely, white, bulky feces, an increase in the number of pellets and meteorism. We are convinced that we have here reproduced the phenomenon of refection.

B. Is refection due to accidental feeding of vitamin B as a contaminant of raw potato starch?

We have attempted to solve this problem in the following ways:

1. Feed one group of rats a diet containing yeast added to a vitamin-B deficient diet in which cooked starch is the carbohydrate. To another group feed an exactly similar diet with the difference that the yeast is added to the starch before cooking. If both groups show essentially the same growth, we are justified in concluding that the cooking of the starch under controlled conditions does not destroy significant amounts of vitamin B that may be present. By inference then, if added vitamin B is not destroyed in the cooking of the starch, we may assume that the cooking does not destroy the vitamin which it has been postulated may be a contaminant

[2] All rats received 2 drops of cod liver oil daily. From the data on food intake and the percentage of yeast in the diet, it is seen that this rat received what is considered an adequate amount of yeast, namely, 0.5 gm. dry yeast daily. In this experiment we thought it important to follow the details of Fridericia's original experiments as closely as possible, but in subsequent experiements we fed the supplements separate from the diet.

of raw starch. If, as indeed it turned out to be, the cooking destroys no vitamin B, the failure of rats to grow on cooked starch cannot be laid to destruction of B in the process of cooking.

We have convinced ourselves that our process of cooking starch leaves the vitamin-B content essentially unchanged (experiment 2, fig. 2).

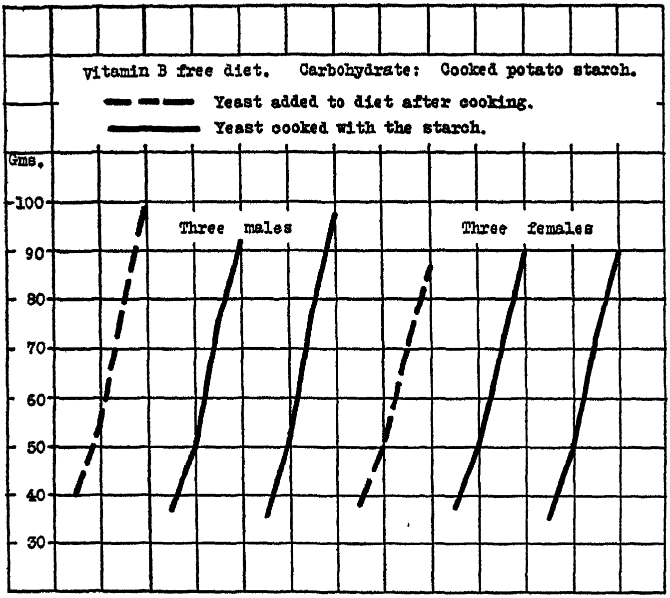

Figure 2

In section A, experiment 1, we used, as stated, a diet made up exactly as used by Fridericia. In all other experiments we made made the following changes—the yeast was added as a separate supplement to the diet—and the casein percentage was correspondingly increased from 20 to 25 per cent of the diet.

Experiment 2. The paired feeding method was used in this experiment. All six rats had identical food intakes.

Figure 2 indicates that cooking starch with yeast added before cooking gives as good a growth response as the adding

of yeast to starch after the cooking. We conclude, therefore, that growth obtained with cooked starch may be compared directly with growth with raw starch as far as the vitamin-B content of the starch is concerned.

2. Secondly, if the growth of refected rats on raw potato starch is to be attributed to vitamin-B contamination of the starch, the growth response should be uniform when no other variable factors are introduced. This is not the case. Experiment 3 (fig. 3) shows growth curves for two of our animals on a vitamin-B deficient diet, indicating the variability in the growth response when all animals are being fed from

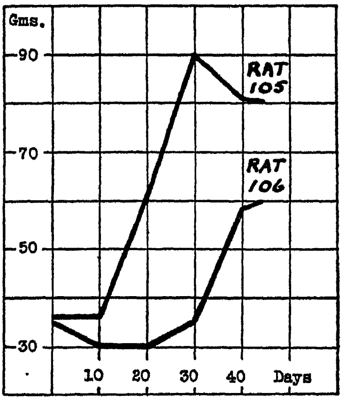

Figure 3

the same lot of diet. Obviously, if the growth obtained was due to vitamin-B contamination of the raw starch, all growth curves should be similar. Rat 105 made rapid weight gains from the tenth to the thirtieth day of the experiment, while rat 106, fed from the same lot of diet, remained stationary in weight. From the thirtieth to the fortieth days of the experiment, the positions were reversed; rat 105 declined in weight while rat 106 gained markedly. These data are representative of other similar results which are omitted for brevity. In order to check all the other constituents of the diet, aside from starch, for the lack of vitamin B we have run continuous deficient controls with cane sugar as carbohydrate

to make sure that our diets are continuously vitamin-B deficient.

In table 1 are assembled some of our data on the gains in weight achieved per gram of food eaten. In general, the positive controls (yeast added to vitamin-B deficient diets) and the refected rats (vitamin-B deficient diets with raw potato starch as the carbohydrate) gained just under 0.5 gm. in body weight per gram of food eaten. Negative controls either lost weight or made insignificant gains. The apparent variability in the

TABLE 1

	FOOD INTAKE	GAIN IN WEIGHT	
		Total	Per gram of food eaten
Experiment 5			
	gm.	*gm.*	*gm.*
Rat 36 (positive control)	128	61	0.47
Rat 37 (refected)	217	96	0.44
Rat 38 (negative control)	49	6	0.12
Rat 34 (positive control)	169	66	0.40
Rat 33 (refected)	86	48	0.55
Rat 35 (negative control)	43	—3	—0.07
Experiment 12			
Rat 89 (positive control)	64	26	0.40
Rat 91 (refected)	73	34	0.46
Rat 93 (negative control)	14	—5	—0.36

amount of food eaten and the gains made by rats under comparable regimes is explained as follows: the data have been used for just the time during which the rats were refected—and as has been shown before by others, the time of onset of refection varies.

C. Examination of the starch of refected feces

1. Separation of raw starch from feces. The dried feces were suspended in water in a cylinder. By successive decantations the starch grains were separated from other materials in the suspension, by their more rapid sedimentation.

2. Salivary digestion. Boiled starch. Using human saliva and boiled starch solutions, incubated at 40° C., we followed the disappearance of the blue color of the starch-iodine reac-

TABLE 2

Pancreatic digestion

1. Control on activity of enzyme preparation

Activity with boiled potato starch as substrate

Enzyme (cc.)	0.6	0.5	0.4	0.3	0.2	0.1	0.05
H_2O (cc.)	0.0	0.1	0.2	0.3	0.4	0.5	0.55
Starch (cc.)	0.6	0.6	0.6	0.6	0.6	0.6	0.6

Incubate at 40° C., cool in ice water at end of 30 minutes.

N/1200 iodine (cc.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Blue color	—	—	—	—	—	—	+

Digestion of boiled starch was complete in all dilutions from 0.6 cc. down to 0.1 cc. enzyme.

2. Activity with raw pure potato starch

Enzyme (cc.)	0.6	0.5	0.4	0.3	0.2	0.1	0.05
H_2O (cc.)	0.0	0.1	0.2	0.3	0.4	0.5	0.55
Dry starch[1] (mg.)	10.0	10.0	10.0	10.0	10.0	10.0	10.0

Incubate at 40° C., cool in ice water at end of 30 minutes, and boil to dissolve any undigested starch; cool.

N/1200 iodine (cc.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Blue color	—	—	—	—	±	+	+

It is seen from this that the enzymatic hydrolysis of raw potato starch is slower than with cooked starch, but digestion is certainly obtained.

3. Activity with raw starch from feces of refected rats

Enzyme (cc.)	0.6	0.5	0.4	0.3	0.2	0.1	0.05
H_2O (cc.)	0.0	0.1	0.2	0.3	0.4	0.5	0.55
Dry starch (mg.)	10.0	10.0	10.0	10.0	10.0	10.0	10.0

Incubate at 40° C., cool in ice water at end of 30 minutes, and boil to render any undigested starch detectable with iodine.

N/1200 iodine (cc.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Blue color	—	—	—	—	±	+	+

[1] Ten milligrams of dry starch is the amount of starch used in the previous test where 0.6 cc. of starch solution was used.

tion as a measure of digestion. A comparison of pure potato starch (such as we used in our diets) with that recovered from refected rat feces showed a complete digestion of both

in 10 minutes. The digestion was more rapid with pure starch than with the starch from refected feces (pure starch digestion was complete in 2.5 minutes). In this connection we should state that our starch from feces was not pure white in color, but we considered it essential that we do nothing more drastic in separating the starch from the feces than suspension in water, to rule out the possibility of our removing from the grains the supposed 70 per cent alcohol-soluble coating described by Fridericia.

Raw starch. Salivary digestion was negative with both raw starches as long as it was followed (60 minutes).

3. Pancreatic digestion. The entire pancreas, and a small section of duodenum, from a freshly killed adult normal white rat was ground up with distilled water, filtered through cloth, and used in 1 per cent suspension. Such preparations are very active.

Using the Whohlgemuth method as a guide, the starch-splitting effectiveness of the preparation was tested in various concentrations of enzyme at 40°C. for 30 minutes (table 2).

It is seen that the raw potato starch separated from refected feces is digested by pancreatic enzyme preparations in a manner exactly similar to the digestion of pure raw potato starch. These experiments were repeated, introducing the single variation of doubling the amount of starch used as substrate, and the digestion of 20 mg. of raw starch from the feces of refected rats was identical with that for 10 mg. There is no escape from the conclusion that in these experiments raw potato starch from refected feces was just as readily digested as pure starch.

DISCUSSION

There is no question but that there is such a thing as refection in rats—and it must be carefully controlled in any biological assays of vitamin B. We have found in the literature what appears to be a case of refection in humans. Langworthy and Deuel ('20) in studying the digestibility of raw potato starch state that, "During this diet practically all the

subjects noted a very excessive formation of gas and frequent intestinal cramps. The quantity of feces voided was very large; a large amount of undigested starch was visible and a strong positive reaction was given with iodine.'' This is certainly a picture resembling the white, bulky, gas-occluded feces in the refected rat. These authors also state that, ''The ingestion of the potato starch caused disagreeable physiological disturbances not noted in the other experiments with raw corn and wheat starches.''

We feel it imperative that the accidental feeding of vitamin B as a contaminant of any food constituent must be ruled out. Our continuous and numerous negative controls throughout the whole time of experimentation in which vitamin-B deficiency was always obtained when cane sugar and corn starch were the carbohydrate components of the diet, justify the view that our diets are really deficient in vitamin B, and when other carbohydrates are substituted for these, there is the necessity of checking the vitamin-B factor of that component only.

The influence of coprophagy on the results has been considered (Page, '32; Guerrant and Dutcher, '32). We are aware of devices intended to prevent coprophagy. We believe that there is no device which can certainly prevent it. If a coprophagy harness is used to prevent animals from ingesting their own feces just as they emerge, they are still able to eat the feces which stick to even very large-mesh flooring of cages, because refected feces are bulky, moist, and stick well to all cage surfaces.

We have run numerous series of rats on varying yeast supplements, and find that growth curves are noticeably lowered when the 0.5 gm. daily supplement is reduced to 0.4 gm. or less. Inasmuch as we do remove a certain quantity of feces from each rat each day, it cannot have re-ingested all the vitamin B excreted.

Guerrant and Dutcher ('32) supplemented their basal vitamin-B deficient diets with feces from vitamin-G deficient rats and obtained gains in weight of only 5 to 7 gm. per week.

Our refected rats gained weight as rapidly as 3 gm. a day. The conclusion from this is that whether coprophagy is practiced or successfully prevented, the refected rat is obtaining a supply of vitamin B that is unobtainable from the feces of the usual vitamin-B deficient rat, and the conclusion is inescapable that the vitamin B is synthesized by the rat under the conditions outlined. Whether the vitamin B is absorbed from the gut directly after synthesis and before the feces are

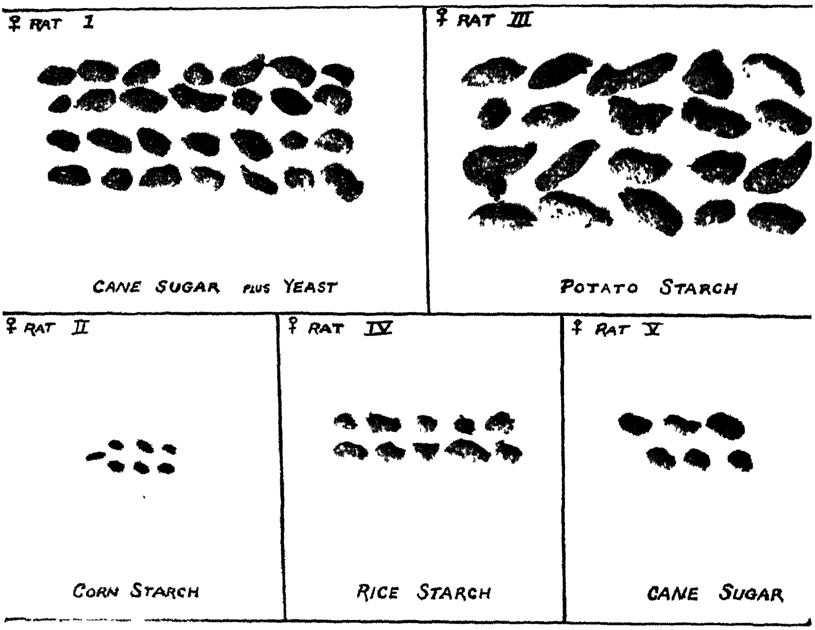

Fig. A Showing the type of feces obtained for 1 day from rat on the respective diets of raw starch (potato, corn, rice) compared with diets where cane sugar served as source of carbohydrate.

voided—or whether the vitamin is obtained, at second hand, so to speak, by re-ingestion of the feces, does not become an issue in deciding whether or not there is such a phenomenon as refection.

Our experiments on the digestibility of the raw potato starch obtained from refected feces indicated no difference in digestibility in comparison with the pure raw potato starch used in the diet.

We have not discussed the recent paper of Guerrant, Dutcher and Tomey ('35) because while it is a further contribution to the subject, it is outside the scope of the present paper.

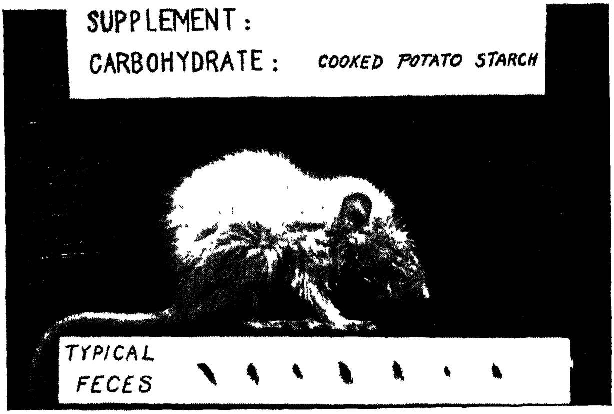

Fig. B Rat 112, 44 days old. Permanently deficient on cooked potato starch, absence of white bulky feces.

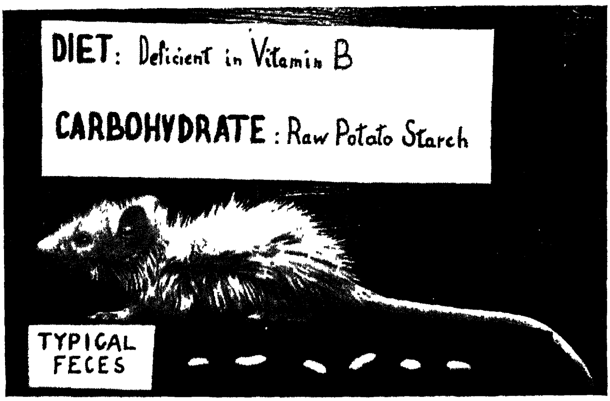

Fig. C Rat 106, 25 days old. Showing the first onset of refection, animal deficient with the first appearance of white, bulky feces which will result in the appearance of refection.

CONCLUSIONS

1. Refection has been confirmed. It can be obtained readily with diets having raw potato starch as the carbohydrate.

2. Growth of refected rats is not to be attributed to erroneously prepared diets containing unsuspected vitamin-B contaminations.

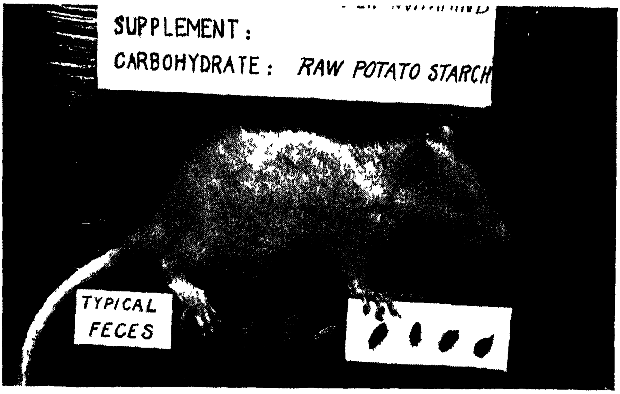

Fig. D Rat 105, 44 days old. Refected rat on diet deficient in vitamin B, showing meteorism, and both bulky white and some normal appearing feces (transition stage from deficient to refected condition). Rat normal in appearance, smaller than rat 104 because it takes a week or so for refection to become established.

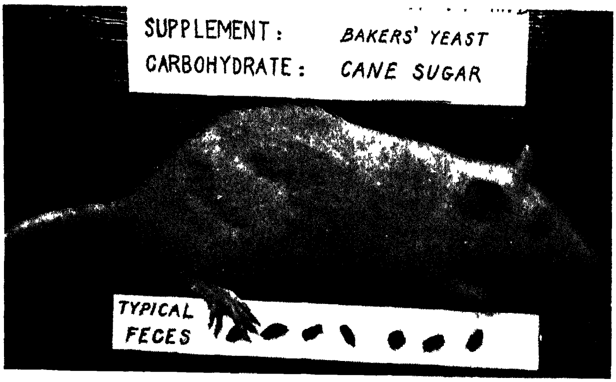

Fig. E Rat 104, 44 days old. Showing normal development on an adequate synthetic diet.

3. Raw potato starch from refected feces, and pure raw potato starch behave similarly in 'in vitro' digestion experiments with pancreatic digestion mixtures.

APPENDIX

Preparation of basic materials for diets deficient in the vitamin-B complex

Purification of commercial casein. Transfer 500 gm. casein to a 6-liter Florence flask; add 0.1 per cent acetic acid (dilute 10 cc. of '99 per cent to glacial' acid to 10 liters of distilled water) until the volume of supernatant acid about equals the volume of moist casein, allowing time for swelling of the casein. There will be enough room in the flask for adequate mixing. Stopper the flask and shake vigorously and frequently for about 4 hours. Allow casein to settle rather firmly and remove the acid wash fluid by decantation. The casein is washed in this manner twice daily for 7 days; each alternate wash stands overnight, and is given a mixing on the following morning before removing the fluid by decantation. When it is inconvenient to change the wash fluid twice in the same day, fourteen separate washings are considered equivalent, the periods to be not less than as given. Filter off the last acetic acid washing.

Return acid-extracted casein to the same flask and add 60 per cent ethyl alcohol (dilute 95 per cent alcohol with water in the ratio of 100 cc. of alcohol to 58 cc. water) bringing the mixture of casein and alcohol up to the same total volume used in the acid extraction. This alcohol extraction is continued for a full 24-hour period—with frequent shaking during the day. The casein receives three such extractions, using filtration instead of decantation after each extraction. One additional and similar extraction is made with 95 per cent alcohol. Filter to near dryness and extract for one similar period with ether.

After filtration from ether, spread out on glazed paper to air dry. When dry, only that part of the casein is used which easily passes a 40-mesh sieve, because we feel that lumpy material may have escaped adequate and uniform treatment. The final product is a finely granular and completely white powder.

Preparation of raw potato starch from potatoes. Starting with 5 pounds of potatoes, peel and remove the eyes completely. After washing, grind very finely in a food grinder. Place mash in a closely woven cotton sack and tie the sack so as to leave sufficient space for subsequent kneading.

Immerse in a pail (enameled) of clear tap water and knead vigorously with both hands until the extraction seems about complete. Kneading under the surface of the water insures appreciably higher yields. Transfer to a glass precipitating jar and pour off the liquid with all suspended material after the starch has stratified on the bottom (about 2 hours).

The moist starch is then suspended in tap water and mixed well. After settling of the starch, the liquid (containing suspended impurities) is decanted. This is continued until the washings are entirely clear. (If the starch is left wet for any extended period, a little chloroform is added.)

The starch is now suspended in water, and the suspension is poured through a 40-mesh, then through a 60-mesh, and finally through an 80-mesh sieve.

After decanting this last wash, which should be clear, spread out the starch on glazed paper to dry. To avoid damage to individual starch grains, the dried product is not screened, but left in a lumpy condition.

Separation of butter fat from butter. Place 2 pounds of butter in a beaker and heat to approximately 50° C. in a water bath until melted.

Transfer to a 2-liter separatory funnel (placed in a ring stand over a source of heat such as will maintain a temperature of about 50° C. in the funnel) and allow to remain until the butter fat separates out (about 1 hour).

Draw off and discard the emulsion in the water layer. Add distilled water (at 50° C.) to the butter fat in the funnel in such amount as will permit of gentle rotary mixing. Washing of the butter fat is accomplished by such frequent additions of water until, after mixing with the butter fat, the water phase is free of turbidity (about 5 to 6 liters of water are usually required). The butter fat receives no further

treatment, and is placed in a glass vessel in the refrigerator. (Our refrigerator maintains a temperature of a few degrees above freezing.) Butter fat, prepared in this way, will keep for months without noticeable deterioration. We have found that it is inadvisable to start with pasteurized butter, due to the difficulty of breaking the emulsions of butter fat.

Preparation of agar. Plain, granular agar is refluxed at boiling temperature with 95 per cent ethyl alcohol. Our impression is that the relative amounts of agar and alcohol may be varied with impunity.

Preparation of dried yeast. Fleishman's bakers' yeast is obtained in pound bricks. Crumble yeast with the hands, spread on glazed paper, and dry at room temperature. Complete the drying on paper-lined trays in an oven at 40°C. Material which does not pass a 20-mesh sieve is discarded.

Preparation of dry diets. Rats will sort out particles of agar from a dry diet and leave this component uneaten. It has been our experience that this can be obviated largely by impregnating the agar with butter fat. Dry casein, carbohydrate and salt mixture are thoroughly mixed. Heat the butter fat and agar together (at 50°C.) and add to the mixture of other dry components. Stirring is carried to the point of obtaining what is apparently a homogenous mixture.

Preparation of diet containing cooked potato starch (78° to 84°C. for 10 minutes). Add 50 cc. of distilled water to 3 gm. of agar in a 100 cc. beaker. Place the agar mixture (beaker covered with watch glass) in a boiling water bath.

Simultaneously, immerse an 800 cc. beaker, containing 52 gm. starch and 470 cc. distilled water, in a water bath kept at a temperature just below boiling. While it is only necessary to stir the agar occasionally, the starch must be kept in constant motion until it gelatinizes at about 65°C. (This is the figure for our own potato starch; the temperature at which starch gelatinizes varies markedly with different starches.) Unless this stage of the preparation is carried out with the utmost care, lumps will form, and the cooking of the starch will not be uniform. Until the temperature of the suspension

reaches 78°C. it is advisable to stir continuously. For the stirring we hold together a thermometer and a heavy glass stirring rod flattened into a paddle at the lower end. Count time from the attainment of a temperature of 78°C. and continue active stirring for 10 minutes. During this time the starch will remain between 78° to 84°C. with the bath temperature maintained at about 98°C.

Remove both the agar and the starch from the water baths. When the agar has cooled to the temperature of the starch, mix them well by pouring the agar into the starch with stirring. When homogenous, add the butter fat and the previously mixed casein and salt mixture and stir until the mixing of the constituents is uniform. Cover with a watch glass and place in refrigerator.

If this procedure is followed closely, the batch will weigh close to 580 gm., and the dry food factor calculated as follows: since the 580 gm. of food so prepared contains 100 gm. of dry material, the amount of food consumed by a rat is calculated as the number of grams of prepared food divided by 5.8, and is recorded thus as grams of dry weight of food. Cooked starch diets are used only up to and including the fifth day after preparation.

Supplements. All rats receive 2 drops of cod liver oil daily by mouth, using a medicine dropper. Where indicated in the data, yeast is placed in a separate dish, and moistened to prevvent loss by scattering. Food is given fresh daily, and the intake weighed daily.

General notes. All rats are kept in individual cages; fresh water is supplied daily, and food and water dishes are of glazed porcelain. With cooked diets, the food dishes are washed daily.

Unless there is an occasion for doing it oftener, feces are collected and the cage cleaned twice in a 5-day period. When a cage is vacated, cleaning is followed by washing with a lysol solution.

Standard cages are used with the exception of special preparation of the bottoms. The bottom pan is covered with an absorbent paper towel. Two ⅜-inch mesh galvanized iron screens are cut to fit the bottom of the pan. These two are separated from each other by a distance of 5 mm., accomplished by three glass rod supports. This arrangement insures that the rat will be dry without allowing the rat to eat any paper. We make no attempt to prevent coprophagy.

Food losses through scattering are minimized by placing a glass Petri dish under the porcelain food cups. This Petri dish is held in place by two slots cut in the standard food cup holder.

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THE COMPARATIVE ANTIRACHITIC EFFICIENCY OF VITAMIN D IN IRRADIATED MILK, METABOLIZED (YEAST) MILK, AND COD LIVER OIL [1,2]

R. M. BETHKE, W. E. KRAUSS, P. R. RECORD AND O. H. M. WILDER
Departments of Animal Industry and Dairy Husbandry, Ohio Agricultural Experiment Station, Wooster

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Several investigators (Mussehl and Ackerson, '30; Massengale and Nussmeier, '30; Russell and Klein, '31; Steenbock, Kletzien and Halpin, '32; Russell, Taylor, and Wilcox, '32, '33; Bethke, Record, and Kennard, '33) have shown that the vitamin D of cod liver oil and that of irradiated ergosterol or irradiated yeast are not identical. This fact came to be established primarily through experiments which demostrated that the chicken responds less effectively to the vitamin D in irradiated ergosterol and irradiated yeast than to the rat equivalent amount of vitamin D in cod liver oil. The report of Waddell ('34) that the provitamin constituent of cholesterol was a substance different from that of ergosterol and that irradiated cholesterol was as efficient for chicks, rat unit for rat unit, as the vitamin D of cod liver oil, gave a possible new interpretation to the vitamin D problem.

The early clinical results (Hess, Lewis, and Rivkin, '30; Barnes, Brady, and James, '30; DeSanctis and Craig, '30) on infants receiving irradiated ergosterol also brought out quan-

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[2] Presented in part before the American Institute of Nutrition, April 10, 1935. Similar data were presented by Haman and Steenbock, Proc. Am. Inst. Nutrition, J. Nutrition, vol. 9, (June) 1935.

titative differences between the vitamin D of irradiated ergosterol and cod liver oil, although to a lesser degree than in case of the chicken. More recent clinical experiments dealing with the ability of vitamin D-rich milks to cure or prevent infantile rickets have yielded results which further complicate the problem. These milks have not only been reported by Hess and associates ('31) as being more efficient than other vitamin D supplements but that they also differ among themselves in efficacy, depending upon the method by which they are enriched with vitamin D. Hess and Lewis ('32, '33) reported that the vitamin D in irradiated milk was more efficacious in the cure of infantile rickets than milk enriched in vitamin D by feeding the cows irradiated yeast. On the contrary, Kramer and Gittleman ('33) and recently Wyman, Eley, Bunker, and Harris ('35), and Gerstenberger, et al. ('35) reported that the two milks were equally efficient clinically when compared on a rat unit basis.

Experimental evidence has been presented which shows that the additional vitamin D in milk or eggs resulting from the feeding of irradiated ergosterol to the cow or chicken is in the same biological form as in the supplement fed. Krauss, Bethke, and Monroe ('32) reported that the vitamin D in butterfat from cows fed irradiated ergosterol was not so efficient for calcification in chicks as an equivalent rat unit of vitamin D from cod liver oil. Likewise, McDonald and Massengale ('32) found that the vitamin D in eggs resulting from feeding large doses of irradiated ergosterol to the laying bird was not so efficient antirachitically for chicks as equivalent rat units of vitamin D from cod liver oil. Similar (unpublished) observations have been made in our laboratory. The present study was undertaken to obtain further data on the above observations and on the comparative response of the infant and chick to the vitamin D in irradiated milk and in milk from cows fed irradiated yeast.

EXPERIMENTAL

The milks came from cows which received the same basal ration of excellent quality alfalfa hay and a grain mixture made up of ground yellow corn, ground oats, wheat bran, and linseed oil meal. In addition, one group received a definite amount of irradiated yeast mixed with the grain portion of the ration. The cows were kept under winter feeding conditions and allowed a small amount of exercise each suitable day in a vegetation-free lot. The irradiated milk was produced by running the milk from the check group of cows over a small surface cooler in a thin film and exposing it to the rays of a carbon arc lamp burning C carbons. The skim milk used in the first experiment and the whole untreated milk used in the second trial were from the check group of cows, i.e., from the same supply used for irradiation. All milks were dried before electric fans at a temperature not exceeding 50°C.

The assays on birds were carried out with single-comb White Leghorn chicks of the same parent stock. They were started on the experimental rations when 1 day old. Fifteen chicks were placed in each group. All groups were brooded indoors in pens equipped with hardware cloth floors. Ash determinations were made on the individual alcohol- and ether-extracted tibiae of twelve representative birds from each group. Calcium (Clark and Collip, '25) and inorganic phosphorus (Briggs, '23) determinations were made on the serum of the pooled blood.

The dried milks used in the first trial were from the same supply of fluid milk that Gerstenberger, et al. ('35) used in their clinical studies. They were thoroughly incorporated at a 25 per cent level in a ration of yellow corn, 42; wheat middlings, 25; commercial casein, 5; calcium carbonate, 2; and sodium chloride, 1. In order to equalize the total milk solids and fats in the rations, 19 per cent dried skim milk and 6 per cent corn oil were included in the rations of lots 1 and 4. The corn oil of the latter group (lot 4) contained cod liver oil to furnish 27 International units per 100 gm. of ration.

The results summarized in table 1 show that vitamin D in metabolized (yeast) milk was not so effective antirachitically for chicks as vitamin D in cod liver oil. Although the comparison between irradiated milk and metabolized (yeast) milk was not clear cut on account of the greater intake of vitamin D in the irradiated milk lot, they nevertheless suggest that the vitamin D in irradiated milk is probably more efficient for calcification in chicks than the factor in metabolized (yeast) milk. It was thought at the start of the experiment that the two milks were approximately equal in rat unit potency; since

TABLE 1

The antirachitic efficacy of vitamin D in metabolized (yeast) milk, irradiated milk, and cod liver oil for chicks

LOT NO.	SUPPLEMENT	VITAMIN D RAT UNITS PER 100 GM. RATION	AVERAGE WEIGHT AT 5 WEEKS	BLOOD ANALYSIS		AVERAGE ASH IN TIBIAE
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	*mg.*	*mg.*	*per cent*
1	19 per cent dried skim milk	0	210.6	8.87	6.73	36.51 ± 0.48
2	25 per cent dried metabolized milk	33	240.4	9.00	4.89	37.48 ± 0.54
3	25 per cent dried irradiated milk	50	266.0	11.74	7.57	47.20 ± 0.24
4	19 per cent dried skim milk Cod liver oil	27	273.8	11.36	7.19	46.92 ± 0.15

earlier preliminary rat assays on similarly prepared fluid milks indicated equal potency. However, when we observed the apparent marked difference in antirachitic efficiency in the chick, the dried milks were assayed on rats for comparative potency. The line test method indicated that the dried metabolized (yeast) and irradiated milks contained 1.33 and 2.0 International units of vitamin D per gram, respectively.

In order further to check the comparative vitamin D potencies of the above milks, they were fed in a prophylactic trial to groups of weanling rats divided evenly as to litter and sex and maintained on the Steenbock and Black rachitic ration.

Appropriate negative and positive control groups were also included. Ash determinations were made on the individual alcohol- and ether-extracted moisture-free femurs at the end of 4 weeks. The data are presented in table 2. It is evident that the irradiated milk had a greater potency than the metabolized (yeast) milk—160 mg. of the irradiated milk daily gave the same per cent of bone ash as 240 mg. daily of the metabolized. On this basis, the comparative potencies of the two milks were on the order of 2 : 3, which is in agreement with the line-test results.

The second chick trial was conducted to obtain a better quantitative comparison between the two kinds of vitamin D

TABLE 2

Comparative vitamin D potencies of dried irradiated milk and dried yeast milk used in first chick experiment (prophylactic)

SUPPLEMENT FED DAILY	NUMBER OF RATS	GAIN IN WEIGHT	AVERAGE BONE ASH IN FEMURS
		gm.	*per cent*
160 mg. dried metabolized milk	8	49.3	40.68 ± 0.31
160 mg. dried irradiated milk	8	51.3	42.84 ± 0.32
240 mg. dried metabolized milk	8	52.8	42.55 ± 0.32
240 mg. dried irradiated milk	8	55.3	45.18 ± 0.24
3 drops cod liver oil	7	35.1	44.57 ± 0.24
None	8	37.3	25.41 ± 0.33

milks and cod liver oil. Since in the first chick experiment 25 per cent of dried metabolized milk did not show a positive antirachitic effect, it was necessary to increase the vitamin D content of this milk without increasing the amount of total milk solids in the ration and thus introduce other complicating factors. Accordingly, a group of cows were fed sufficient irradiated yeast to produce a highly potent milk. The irradiated milk was produced as in the first trial. Whole dried milk from the check group of cows was used in place of dried skim milk in order to have a uniform intake of total milk solids and a direct comparison between the efficacy of the vitamin D in the two kinds of milk due to individual treatment.

The dried milks were first assayed on rats by the line-test and bone-ash methods so that known amounts of vitamin D could be incorporated in the chick rations. The results of the line-test showed that the dried metabolized (yeast) milk and irradiated milk contained 4.4 and 2.2 International units, respectively, per gram. The vitamin D content of the dried check milk was so low that it was considered negligible. The results of the prophylactic assay on the milks are presented in table 3. It is apparent that the dried metabolized (yeast) milk contained approximately again as much vitamin D as

TABLE 3

Comparative vitamin D potencies of dried irradiated milk and dried metabolized (yeast) milk used in second chick experiment (prophylactic)

SUPPLEMENT FED EVERY OTHER DAY	NUMBER OF RATS	GAIN IN WEIGHT	AVERAGE BONE ASH IN FEMURS
		gm.	*per cent*
None	7	31.6	25.71 ± 0.59
165.0 mg. dried skim milk	7	40.1	25.78 ± 0.72
225.0 mg. dried whole check milk	7	38.1	26.43 ± 0.49
75.0 mg. dried metabolized milk 112.5 mg. dried skim milk	7	47.4	40.03 ± 0.74
150.0 mg. dried metabolized milk 52.5 mg. dried skim milk	7	46.6	45.26 ± 0.67
150.0 mg. dried irradiated milk 52.5 mg. dried skim milk	7	47.4	41.93 ± 0.86
225.0 mg. dried irradiated milk	7	46.3	44.06 ± 0.55
3 drops cod liver oil, daily	7	41.4	46.07 ± 0.52

the irradiated milk, since 75 mg. of the metabolized milk and 150 mg. of the irradiated milk gave about the same per cent of bone ash, thus substantiating the line-test evaluations.

In the chick experiment the dried milks were incorporated in the same basal ration used in the first trial. The total solids were kept the same in all rations by replacing an equivalent amount of dried whole check milk with the milks to be assayed. Two groups receiving in addition to the basal ration containing the dried check milk 11 and 27 International units of vitamin D from cod liver oil per 100 gm. of ration

were included for comparative purposes. The cod liver oil was diluted with corn oil so that 1 per cent supplied the desired unitage. The experiment was terminated at 43 days.

Table 4 shows conclusively that the vitamin D in metabolized (yeast) milk is decidedly less efficient antirachitically for

TABLE 4

The comparative antirachitic efficiency of vitamin D milks and cod liver oil for chicks

LOT NO.	SUPPLEMENT	VITAMIN D RAT UNITS PER 100 GM. RATION	AVERAGE WEIGHT AT 43 DAYS	BLOOD ANALYSIS		AVERAGE ASH IN TIBIAE
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	*mg.*	*mg.*	*per cent*
1	25.0 per cent dried whole check milk	0.0	311.1	8.46	6.32	37.44 ± 0.34
2	25.0 per cent dried whole check milk Cod liver oil	11.0	428.1	11.28	8.15	48.13 ± 0.23
3	25.0 per cent dried whole check milk Cod liver oil	27.0	383.4	11.49	8.43	47.73 ± 0.24
4	12.5 per cent dried metabolized milk 12.5 per cent dried whole check milk	55.0	337.9	9.50	6.80	38.54 ± 0.68
5	25.0 per cent dried metabolized milk	110.0	335.4	9.82	6.64	42.77 ± 0.57
6	5.0 per cent dried irradiated milk 20.0 per cent dried whole check milk	11.0	387.9	11.43	8.18	48.34 ± 0.34
7	12.5 per cent dried irradiated milk 12.5 per cent dried whole check milk	27.5	410.5	11.07	8.18	48.35 ± 0.26

the bird than the rat equivalent amount of vitamin D from cod liver oil or irradiated milk; more than ten times the rat unitage of vitamin D in metabolized milk than in irradiated milk or cod liver oil being required to give approximately the same per cent of bone ash. It is further indicated that the vitamin D of irradiated milk and cod liver oil are equally efficient. These findings are in accord with those of Haman and

Steenbock ('35) and follow the observation of Waddell ('34) that vitamin D of irradiated cholesterol and cod liver oil are equivalent for the chick. The results obtained with the metabolized (yeast) milk also substantiate the reports of Krauss, Bethke, and Monroe ('32) and McDonald and Massengale ('32) that the animal or bird does not change one biological form of vitamin D into another.

The marked difference in the response of the chick to vitamin D from the two antirachitic milks is of especial interest, since milks from the same supply were found by Gerstenberger, et al. ('35) to possess no practical difference in antirachitic efficacy for rachitic infants when fed on an equivalent rat unit basis. It appears, therefore, that the infant and chick vary greatly in their response to equivalent rat unit intakes of vitamin D in irradiated milk and metabolized (yeast) milk.

CONCLUSIONS

It required more than ten times the rat equivalent amount of vitamin D in metabolized (yeast) milk than in irradiated milk to produce the same antirachitic effect in chicks.

Equivalent rat units of vitamin D from cod liver oil and irradiated milk were equally efficient, antirachitically, for the chicken.

The data indicate that the vitamin D in milk resulting from feeding irradiated yeast to the cow (metabolized milk) is in the same biological form as that fed to the animal.

Since rat equivalent amounts of vitamin D in metabolized (yeast) milk and irradiated milk were found equally efficacious for the rachitic infant and not for the chick, it is concluded that the infant and chick vary greatly in their response to the forms of vitamin D in these two types of milk.

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THE EFFECT OF DIGESTIBILITY UPON THE AVAILABILITY OF IRON IN WHOLE WHEAT

ELLA McCOLLUM VAHLTEICH, MARY SWARTZ ROSE AND GRACE MACLEOD

Nutrition Laboratory, Teachers College, Columbia University, New York City

ONE FIGURE

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In former papers (Rose et al., '32, '34) we have called attention to the superior results in hemoglobin regeneration which we have observed when anemic rats are fed whole wheat, as compared with results on a solution of the ash of the same amount of wheat. These findings regarding the availability of the iron in the natural food appear to be at variance with those of Elvehjem, Hart and Sherman ('33), but the latter authors have made no comparison between the effect of wheat and of the same amounts of iron and copper as in the wheat in the form of ferric chloride and copper sulphate. They fed young anemic rats 3 gm. of whole wheat yielding 0.15 mg. of iron, supplementing this with 0.05 mg. of copper; but ferric chloride was fed at levels of 0.5 and 0.3 mg. of iron and copper sulphate at a level of 0.05 mg. of copper with 0.04 mg. of manganese in addition. They say, "It is readily seen that the rate of hemoglobin formation produced by the addition of 3 gm. of wheat or oats to a milk diet supplemented with copper is much inferior to that produced by 0.5 mg. of Fe or even 0.3 mg. of Fe supplied as ferric chloride." This would naturally be expected since the wheat furnished only 30 to 50 per cent as much iron as was given in the form of ferric chloride. These findings would not seem to give any indication of the relative availability of the iron in the two forms.

It has been suggested by Elvehjem, Hart and Sherman ('34), that the differences reported with regard to the efficiency of wheat in hemoglobin regeneration may be due to the degree to which the experimental animals are depleted. We have, in the present series, subjected our animals to a more severe anemia than previously, although it has been our experience that for the testing of foods it is not desirable to go too far in this direction, since animals very severely depleted show a high mortality and give other evidences of poor physiological condition. They frequently lose appetite and fail to respond as quickly to an improved diet as animals which have a slightly higher hemoglobin level and are otherwise apparently normal. In view of these difficulties with very anemic animals, it seemed to us quite likely that the failure of very young and very anemic animals to respond to the iron in wheat might be due to difficulty in digestion of wheat. Hence in this study we have used wheat predigested with Taka diastase till the starch was completely dextrinized to furnish iron in amount equal to the raw wheat (fed in the form of finely-ground flour).

In making comparisons of this sort it is desirable to have the iron intake considerably under the optimum amount for normal regeneration. We have therefore used 3 gm. of whole wheat, yielding 0.1 mg. of iron and 0.02 mg. of copper, and have paralleled the wheat experiment with one in which the same amounts of iron and copper were fed as ferric chloride and copper sulphate respectively.

Until the young were 2 weeks old the mother rats were fed our stock ration consisting of one-third whole milk powder, two-thirds whole wheat, sodium chloride equal to 2 per cent of the wheat with a few grams of lean beef daily. The third week all received whole milk powder, but the whole wheat was given only to the mothers, who were removed from their young for several hours daily to receive the wheat. Three litters of young, thirty-one animals, were weaned at 3 weeks of age and fed exclusively on fresh whole Guernsey milk, brought in glass containers directly from the cow to the laboratory.

After 33 to 38 days on this ration, twenty-four rats had hemoglobin values between 2.2 and 3.6 gm.; and four rats, values which ranged between 4.2 and 4.5 gm. The remaining three animals, which after 44 to 46 days still had hemoglobin values of over 5 gm., were discarded. One animal died 6 days after being reduced to a hemoglobin value of 3 gm., and two others with values of 3.0 and 4.2 gm. at 57 and 53 days respectively were too weak to respond when fed the supplementary source of iron and died, one after 6, the other after 3 days of supplementary feeding.

Twenty-four animals, all with hemoglobin values between 2.2 and 3.6 gm. per 100 cc. of blood with the exception of two animals which had values of 4.4 and 4.5 gm. respectively, were divided into three lots carefully matched as to litter, age, sex, weight and hemoglobin level. These were fed fresh whole milk daily for 6 weeks, lot 1 receiving in addition 3 gm. of whole wheat finely ground; lot 2, the same amount of iron in the form of 2.7 gm. of wheat predigested till no starch could be detected with iodine; lot 3, 0.1 mg. iron as $FeCl_3$ and 0.02 mg. copper as $CuSO_4$, equivalent to the iron and copper in 3 gm. of whole wheat. At the end of 6 weeks, the total gain in hemoglobin for the undigested whole wheat averaged 7.6 gm. per 100 cc. of blood; for the predigested whole wheat, 9.6 gm.; and for the mineral supplements, 7.0 gm. The data are summarized in table 1 and in the rates of hemoglobin regeneration in figure 1.

These animals required approximately 5 weeks for depletion after weaning at age of 21 days. This is longer than the period reported by Elvehjem and his associates. The difference may be due to a higher iron content of the diet used by us for the mothers. Our animals weighed approximately twice as much at depletion, and being relatively stronger, were able to consume easily and digest well 3 gm. of whole wheat daily. Nevertheless, the improved utilization of the iron when the wheat was predigested, as indicated by a 26 per cent greater gain in hemoglobin in 6 weeks, may be taken as evidence that digestibility is a factor to be considered in the utilization of iron by young anemic rats.

TABLE 1

Age, weight and hemoglobin of anemic rats fed 3 gm. whole wheat, the same amount predigested, and iron and copper to equal that in the wheat as $FeCl_3$ and $CuSO_4$

	3 GM. WHOLE WHEAT	2.7 GM. PREDIGESTED WHOLE WHEAT	0.1 MG. Fe (AS $FeCl_3$) 0.02 MG. Cu (AS $CuSO_4$)
Average age at depletion	56 days	57 days	57 days
Average weight at weaning (21 days)	33 gm.	32 gm.	32 gm.
Average weight at depletion	80 gm.	78 gm.	79 gm.
Average weight at end	206 gm.	197 gm.	161 gm.
Average hemoglobin per 100 cc. of blood at beginning	3.3 gm.	3.1 gm.	3.3 gm.
Average hemoglobin at end of 6 weeks feeding	10.9 gm.	12.7 gm.	10.3 gm.
Average gains in hemoglobin in 6 weeks	7.6 gm.	9.6 gm.	7.0 gm.

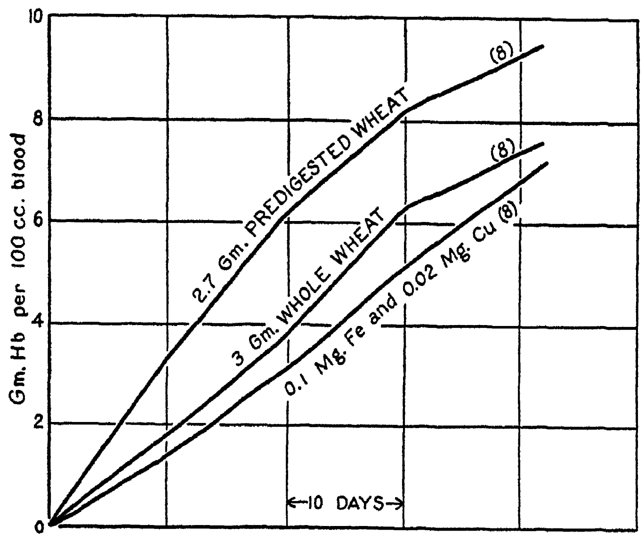

Fig. 1 Average weekly increases in hemoglobin from feeding the same amount of iron (0.1 mg.) and of copper (0.02 mg.) in form of whole wheat, predigested whole wheat, and iron and copper as $FeCl_3$ and $CuSO_4$. Figures in parentheses indicate the number of animals.

Failure to attain as good gains on the ash of whole wheat as on the original grain has been reported in a previous paper (Rose, et al., '32). Feeding the ash of 6 gm. of whole wheat resulted in 28 per cent less increase in hemoglobin in 6 weeks than occurred on feeding the whole wheat itself. In the present study the hemoglobin increase on the mineral supplements was only 8 per cent less than on the whole wheat but 21 per cent less than on the predigested wheat.

The findings with regard to gains in hemoglobin on 3 gm. of whole wheat are in good accord with our former report in which eleven animals, averaging hemoglobin 5.4 gm. and an age of 83 days at the end of the depletion period, showed an average gain of 6.7 gm. hemoglobin in 6 weeks. This confirms our view that in case of iron, as of protein, since it is not possible to deplete the body completely, any hemoglobin level low enough to afford opportunity for a considerable increase before reaching the full normal value is suitable for studies of the efficiency of foods in hemoglobin regeneration.

SUMMARY

Young rats depleted to hemoglobin levels averaging 3.3 gm. per 100 cc. of blood at 8 weeks of age were fed as supplements to fresh whole milk (1) whole wheat (3 gm.), yielding 0.1 mg. of iron and 0.02 mg. copper; (2) whole wheat (2.7 gm.) with all the starch dextrinized, furnishing the same amounts of iron and copper as (1); (3) 0.1 mg. of iron as $FeCl_3$ and 0.02 mg. copper as $CuSO_4$. Hemoglobin regeneration was best on the predigested wheat, next best on the natural wheat finely ground, and poorest on the mineral supplements, the gains in 6 weeks amounting to 9.6 and 7.6 and 7.0 gm. of hemoglobin per 100 cc. of blood respectively. The ease of digestion of the dextrinized wheat we believe to have been the factor causing the better hemoglobin regeneration on this than on the wheat finely ground but not predigested.

The gains in hemoglobin on 3 gm. of whole wheat in this series with animals depleted to an average hemoglobin level of 3.3 gm. of hemoglobin per 100 cc. of blood are in good agreement with those for animals in a previous series depleted to a level of 5.4 gm. As long as the level at depletion is sufficiently low to allow for considerable increase before reaching the normal hemoglobin level, we believe tests for hemoglobin regenerating values of foods will be reliable.

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PROTEIN UTILIZATION AS AFFECTED BY THE PRESENCE OF SMALL AMOUNTS OF BRAN OR ITS FIBER

ESTHER H. FUNNELL, ELLA McC. VAHLTEICH, SADIE O. MORRIS, GRACE MACLEOD AND MARY S. ROSE

Nutrition Laboratory, Teachers College, Columbia University, New York City

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The concept of an adequate diet was exceedingly simple before the discovery of the vitamins and of the important roles in nutrition of a whole category of mineral elements. 'Balance' between the non-nitrogenous fat and carbohydrate on the one hand and the protein on the other, reduced to an isodynamic basis, was conceived to fulfill the conditions of good nutrition. With this idea dominant, the availability of the protein in any edible material became one of the chief criteria of its value as a food. Today we not only recognize many other substances in food besides protein as of prime nutritional significance, but also appreciate that the protein requirement of human beings is much lower than was formerly supposed. Hence the student of the American dietary is usually more concerned lest the supply of the vitamins and of certain mineral elements be inadequate than lest there be a real deficiency of protein. Foods must now be evaluated for the various nutrients which they may yield. Thus the tomato, once viewed askance by the physiologist because neither a good source of energy nor of protein, has come to be recommended as a staple in the diets of the poorest people because of its richness in vitamin C. Similarly there has been some change of emphasis with regard to the contributions made to the dietary by the cereal grains. While they remain of the greatest significance the world over as

cheap fuel for the human machine, they also constitute a most economical source of protein, iron and vitamin B. McCarrison ('28) has pointed out that in India the dependence of the mass of the population upon cereal food as a source of energy gives significance to differences in grains in other values besides calories. Rice even when not deprived of its bran coats is inferior to wheat in its power to support growth.

It is well known that the addition of cellulose to any ration lowers the coefficients of digestibility of its proteins, since cellulose calls out an increased secretion from the intestinal tract and the nitrogen in this secretion is not all reabsorbed but passes out in the feces. This effect was studied by Mendel and Fine ('12), who added 3 gm. of agar-agar and 7 gm. of bone ash to meat powder yielding 4.6 to 4.9 gm. nitrogen and found in dogs an increase in fecal nitrogen amounting to 60 per cent or more. Some increase of fecal nitrogen must, therefore, be expected when any cellulose is added to the diet, but for the moderate amounts of roughage which serve to keep the diet of the normal adult satisfactory as to laxation, this loss is not as great as many seem to think. Snyder ('01) in a series of three digestion experiments reported a difference of approximately 5 per cent between the coefficient of digestibility of the proteins of white flour and the proteins of the same flour to which had been added 14 per cent of finely ground bran to make it equivalent to graham flour. Newman and others ('12) in a study of white and whole wheat flour continued over 2 weeks found the average coefficient for the proteins of white flour 89.3 per cent while that for whole meal was 85.9 per cent, a difference in this relatively long experiment of only 3.4 per cent. Holmes ('19) summarizing various tests made by the U. S. Department of Agriculture, reports the average coefficient of digestibility for protein of white flour to be 88.1 per cent (thirty-one experiments) and for that of graham flour to be 76.9 per cent (twenty-four experiments), a difference of 11.8 per cent. Holmes ('19) investigated the digestibility of a diet in which the total bran averaged in one series 155 gm., finely ground, and in another 132 gm., coarsely ground. The bran was added to a ration

of potato, fruit, butter and sugar. The diet furnished on the average only 24 gm. of protein and the alimentary tract was so stimulated that the output of nitrogen occasionally exceeded the intake. The coefficient of digestibility for the protein of the diet was 37 per cent when fine bran was eaten and 35.8 per cent when coarse bran was substituted for fine. This was far too large an amount of bran to indicate what happens when only a small amount is added to the diet, or its influence when separated from the other portions of the grain and prepared for human consumption by thorough steaming and toasting.

From the commercial point of view the separation of endosperm, bran and germ has certain advantages. The perishable embryo, one of our richest and cheapest sources of vitamin B, can be suitably preserved and used to restore to the diet this factor which tends to be low when whole grains are exchanged for highly milled cereals. The bran not only furnishes roughage in a convenient form (Rose et al., '32) but is a good source of vitamin B (Rose, Vahlteich and Funnell, '32) and of iron (Rose, Vahlteich and MacLeod, '34) and contains proteins of excellent quality. According to Jones and Gersdorff ('25) 22 per cent of the nitrogen of the wheat kernel is in the bran. Murphy and Jones ('26) fed seventy young rats diets containing 70 per cent of bran, plus 12 per cent starch and 14 per cent butter fat, with suitable mineral and vitamin supplements and found the bran proteins efficient for growth for 6 weeks, gains being 1.83 gm. per gram of protein eaten.

It seemed worth while to find out what the effect upon the output of nitrogen of human beings would be when a small amount of prepared bran (approximately 1½ ounces) was added daily to a simple mixed diet, in comparison with the pure fiber from a similar portion of bran supplemented by crystalline vitamin B hydrochloride[1] to equal the amount of vitamin B present in the original bran.

Two healthy young women served as subjects. The experimental periods were each 9 days in length and a preliminary

[1] Kindly furnished by Mr. R. R. Williams.

period of 3 days on the experimental ration without collection of samples preceded each of the four periods. The basal diet consisted of milk, sugar, lactose, cornstarch, butter fat, cream, grape juice and apples. To this ration was added in the first period prepared bran, 45 gm. for one subject and 54 gm. for the other, the amount being adjusted so that the amount of nitrogen per kilo of body weight would be the same for each subject. Since in a study formerly made on animals (Rose et al., '32) it had been found that a small amount of bran added to a diet deficient in vitamin B did not induce the same laxative effect as when added to one in which vitamin B was liberal, the equivalent of the amount of vitamin B present in the bran was added in the periods in which pure fiber or no fiber was used, as an extra precaution to keep conditions as nearly uniform as possible. In the second period, pure bran fiber replaced the original bran. In the third period, in addition to the pure fiber, one subject received daily 76 units of vitamin B in the form of pure hydrochloride and the other subject 90 units daily. In the fourth period, the basal ration was taken with no addition except the vitamin B in the same amounts as in the third period. The starch, lactose and butter fat were made into wafers and baked till crisp. These served as a substitute for bread and were very palatable.

The total protein intake was limited to 0.5 gm. per kilo of body weight in order to keep the intake close to the minimum, and afford a better opportunity to observe the effect of the bran on the nitrogen balance. Also it served to keep the percentage of the total protein derived from bran fairly high, this amounting to 22 per cent throughout with the exception of the first 3 days of period I for subject M when it furnished 18.6 per cent.

All foods were weighed before ingestion and suitable samples were analyzed for total nitrogen by the Kjeldahl method. Urine and feces were collected daily, carmine being used as a marker for the feces. Analyses of urine and feces were made by 3-day periods. The food intake and the nitrogen content of each of the foods used are shown in table 1.

TABLE 1

Daily food intake and nitrogen content of diet

	Subject F weight 58 kg.			Subject M weight 68 kg.		
	Food	Intake	N in food	Food	Intake	N in food
		gm.	*gm.*		*gm.*	*gm.*
Period I	Milk	700	3.69	Milk	880[1]	4.54
	Cream	70		Cream	70	
Basal diet	Butter fat	40		Butter fat	20	
plus bran	Apple	250	0.04	Apple	250	0.04
	Lactose	75		Lactose	75	
	Bran	45	1.05	Bran	45[2]	1.05
	Sucrose	50		Sucrose	50	
	Cornstarch	60		Cornstarch	40	
	Grape juice	250		Grape juice	200	
	Total		4.78	Total		5.63
Period II	Milk	875	4.54	Milk	1050	5.53
	Cream	70		Cream	70	
Basal diet	Butter fat	40		Butter fat	30	
plus bran	Apple	250	0.04	Apple	250	0.04
fiber	Lactose	55		Lactose	20	
	Bran fiber	3.5	0.00	Bran fiber	3.5	0.00
	Sucrose	50		Sucrose	40	
	Cornstarch	60		Cornstarch	50	
	Grape juice	250		Grape juice	200	
	Total		4.58	Total		5.57
Period III	Milk	875	4.56	Milk	1050	5.39
	Cream	70		Cream	70	
Basal diet	Butter fat	40		Butter fat	40	
plus bran	Apple	250	0.04	Apple	250	0.04
fiber and	Lactose	55		Lactose	55	
vitamin B	Bran fiber	3.5	0.00	Bran fiber	3.5	0.00
	Sucrose	50		Sucrose	50	
	Cornstarch	60		Cornstarch	60	
	Vitamin B (75 units)			Vitamin B (90 units)		
	Total		4.60	Total		5.43
Period IV	Milk	875	4.50			
	Cream	70				
Basal diet	Butter fat	40				
plus vitamin B	Apple	250	0.04			
	Lactose	55				
	Sucrose	50				
	Cornstarch	60				
	Grape juice	250				
	Total		4.54			

[1] Changed after first 3 days to 845 gm.

[2] Changed after first 3 days to 54 gm.

For subject F, who was tall and thin, the allowance of 0.5 gm. of protein per kilo of body weight was slightly under requirement, as shown by a deficit of 0.92 gm. of nitrogen daily in the period when no bran or fiber was ingested and also by the fact that in each period the urinary nitrogen exceeded the nitrogen intake slightly. For subject M who had a heavier build and more body fat, the allowance slightly

TABLE 2

Daily nitrogen intake and output

	SUBJECT F						SUBJECT M					
	N in food	N in urine	N in feces	N output	N balance	Per cent N in feces	N in food	N in urine	N in feces	N output	N balance	Per cent N in feces
	gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.	
Period I Basal diet plus bran	4.78	4.98	1.29	6.27	—1.49	27	5.67	4.99	1.27	6.26	—0.59	22
Period II Basal diet plus bran fiber	4.59	4.80	0.92	5.72	—1.13	20	5.57	5.31	0.85	6.16	—0.59	15
Period III Basal diet plus bran fiber and vitamin B	4.61	4.70	0.86	5.56	—0.95	19	5.43	4.32	0.99	5.31	+0.12	18
Period IV Basal diet plus vitamin B	4.54	4.73	0.73	5.46	—0.92	16						

exceeded requirement, the urinary nitrogen falling short of the intake in all periods, and a slight positive balance of 0.12 gm. appearing in the period when fiber and vitamin B were taken. The daily balances are shown in table 2. Subject M was obliged to withdraw from the experiment at the close of the third period, on account of transfer of residence to another part of the country.

That the prepared bran was more laxative than its fiber, which had been finely subdivided by the vigorous treatment to which it had been subjected in purification, is clearly shown

TABLE 3

Weight of feces

	DAYS	SUBJECT F		SUBJECT M	
		Moist weight	Dry weight	Moist weight	Dry weight
		gm.	*gm.*	*gm.*	*gm.*
Period I Basal diet plus bran	1–3	380	122	430	91
	4–6	302	82	412	101
	7–9	370	91	505	115
	Total	1052	295	1347	307
Period II Basal diet plus bran fiber	1–3	227	62	229	58
	4–6	175	54	280	66
	7–9	230	55	232	55
	Total	622	171	741	179
Period III Basal diet plus bran fiber and vitamin B	1–3	209	60	297	74
	4–6	167	51	354	69
	7–9	274	52	298	65
	Total	650	163	949	208
Period IV Basal diet plus vitamin B	1–3	241	47		
	4–6	166	43		
	7–9	109	32		
	Total	516	122		

TABLE 4

Percentage excess of total weight of feces of bran period I over other periods (II, III, IV)

		SUBJECT F	SUBJECT M
		per cent	*per cent*
Excess in period I over period II	Moist feces	70	80
	Dry feces	70	70
Excess in period I over period III	Moist feces	61	42
	Dry feces	81	48
Excess in period I over period IV	Moist feces	104	
	Dry feces	142	

by comparing the fecal output in each period. The weights of feces, both moist and dry, are shown in table 3 and the percentage by which the weight of feces in the bran period exceeds that of each of the other periods in table 4. The

greatest difference in case of subject F is between the bran period and that period when no bran or fiber was taken, the weight of the moist feces in the former period being 104 per cent and of the dry feces 142 per cent of that of the latter period. The differences in the fiber periods (II and III) average 65 per cent for the dry feces. In case of subject M the differences are similar.

The effect on the proportion of the nitrogen intake excreted in the feces is shown in table 2. On closely comparable intakes, subject F excreted 27 per cent of the total intake in the feces and subject M 22 per cent when the diet was supplemented by the prepared bran, while on the two diets with the pure fiber, subject F excreted 19 and 20 per cent, and subject M, 15 and 18 per cent, respectively. The lowest percentage was, of course, in the period with neither bran nor fiber (subject F), 16 per cent. The periods with the added fiber but no bran protein show the mechanical effect of the fiber on the nitrogen excretion. Since vitamin B in the quantities added apparently made no difference, periods II and III may be averaged and the coefficient of digestibility of the protein estimated as 81 per cent for subject F and 83 per cent for subject M. The coefficient for the diet with the added bran is 73 per cent for subject F and 78 per cent for subject M. The difference must be assumed to represent loss of bran protein, and the coefficients of digestibility of this are therefore 45 per cent for subject F and 59 per cent for subject M.

The inclusion of the fiber, which had little direct laxative effect, as shown by the total fecal output in periods II and III reduced the coefficient of digestibility of the diet as a whole only about 3 per cent in case of subject F while the bran reduced it 11 per cent. Part of this probably represents unabsorbed bran proteins and part extra metabolic products due to the greater laxative effect of the bran.

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INFLUENCE OF THE RATION ON THE VITAMIN C CONTENT OF MILK [1]

W. H. RIDDELL, C. H. WHITNAH, J. S. HUGHES AND H. F. LIENHARDT
Kansas Agricultural Experiment Station, Manhattan

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The subject of this paper is a controversial one. Early literature which has been quoted in most texts and reviews of the subject supports the conclusion that the ration has a marked influence on the vitamin C ascorbic acid content of milk. In a recent review (associates of L. A. Rogers, '35), it is stated, that "The evidence although conflicting leaves little doubt that the supply of vitamin C in the diet of a lactating animal affects the concentration of it in its milk."

As early as 1921, studies at this station (Hughes and others, '21) indicated little relation between the ration and the antiscorbutic properties of the milk. Since these findings were not in agreement with results reported elsewhere (Barnes and Hume, '19; Hart et al., '20; Dutcher et al., '20; Hess et al., '20), the work was repeated under carefully controlled conditions with essentially the same results (Hughes et al., '27).

Subsequent to the publication of this work, a more satisfactory method for assaying the vitamin C content of milk has been developed in other laboratories. By the use of these new methods, MacLeod ('27) and Schwartze, Murphy and Hann ('30) found the vitamin C potency of winter milk from ensilage fed cows to be higher than any heretofore reported. In the latter laboratory such milk was found to be almost

[1] Contribution no. 100, Department of Dairy Husbandry, no. 192, Department of Chemistry, and no. 63, Department of Veterinary Medicine.

if not actually as potent as the best summer milk obtained from cows on pasture. Kieferle and Zeiler ('26) also have reported that the milk of ensilage fed cows is richer in growth promoting and antiscorbutic agents than that of cows kept on the usual winter food. On the other hand, de Wildt and Brouwer ('30) could find no seasonal variation in the vitamin C content of cows' or goats' milk; while more recently Wijngaarden ('34), using titration methods, observed no noticeable change in the quantity of vitamin C after adding silage to the cows' ration.

In view of the lack of agreement in the results reported from different laboratories it seemed desirable to make further study of the problem using the more desirable procedure for biological assay and one of the more recently developed chemical tests.

EXPERIMENTAL

A. Biological tests

In 1932, a more extensive series of tests was run using the more approved technic. The basal ration fed the guinea pigs was as follows: skim milk powder, 30 per cent; rolled oats and wheat bran, 59 per cent; butterfat, 8 per cent; cod liver oil, 2 per cent; and salt 1 per cent.

The skim milk powder was heated for 4 hours at 110°C. with frequent stirring to insure as complete destruction of vitamin C as possible. This ration with the addition of orange juice has proved adequate for growth and the prevention of scurvy.

The guinea pigs used in this experiment were obtained from the animal genetics laboratory at this station. With ample numbers available care was taken to select only animals which proved satisfactory milk drinkers. Animals of approximately 300 gm. weight were chosen where possible.

Each guinea pig was maintained in a separate cage with wire bottom. The supply of the basal ration kept before each animal was replenished every 3 days and an accurate record of feed consumption maintained throughout the experiment. The animals were weighed at intervals of 3 days.

The milk was furnished in iron dishes. Morning and evening milk from the different lots of cows was fed within 2 hours of production. All milk was fed at the 40 cc. level with equal division between the two milkings. Furthermore, if any milk was unconsumed at the end of 2 hours, the pig was fed an equivalent amount of fresh milk by pipette. In this way total milk consumption was assured within a relatively short time after feeding. Since the guinea pig is not a heavy consumer of liquid, the results of earlier work may have been complicated by the lack of complete consumption.

The milk tested was from cows on several carefully controlled rations. Lot I had access to good brome and blue grass pasture and received sorghum silage and a grain mixture in addition. Lot II was fed a typical winter ration of alfalfa hay, sorghum silage, and a grain mixture; while lot III received the same ration except that silage was eliminated. Lots II and III were maintained in dry lot throughout the experiment. Lot IV consisted of a group of cows which had been in dry lot over a year, receiving a dry ration of prairie hay, beet pulp, corn, oats, and blood meal.

There were two cows in each lot, an Ayrshire and a Holstein, with the exception of lot IV, which consisted of two Holsteins. The average production and butterfat percentage of the different lots was fairly well balanced, though the butterfat percentage of lot II averaged nearly 1 per cent higher in the final 2 months.

The milk from the cows in each lot was mixed before each feeding and a composite sample used for feeding.

Close clinical observation was maintained on all guinea pigs throughout the experiment. At least two of the authors made daily examination of each animal for symptoms of scurvy. At death or on conclusion of the 90-day period autopsy findings were recorded on the basis of the scurvy score as developed by Sherman and his co-workers ('31).

Table 1 shows the results obtained from feeding the basal ration. It will be noted that first clinical signs of scurvy appeared in 16 to 17 days, while in the two lots tested, the

average days to death ranged from 31 to 36 days with a high scurvy score. This demonstrates the deficiency of the basal ration in vitamin C and the results obtained through its use are in accord with the findings of some of the more recent work in other laboratories.

Since several comparatively recent studies, previously mentioned, have attributed the relatively high vitamin C content of milk from stall fed cows to the silage which they were receiving, a test was made of the antiscorbutic properties of the silage which the cows were fed in this experiment, through direct feeding to a group of guinea pigs. Comparison of the results for average days to first signs of scurvy, average days

TABLE 1

Vitamin C content of silage

RATION	GUINEA PIGS (NO.)	AVERAGE INITIAL WEIGHT	AVERAGE MAXIMUM WEIGHT	AVERAGE FINAL WEIGHT	FIRST SIGNS SCURVY	AVERAGE DAYS TO DEATH	SCURVY SCORE
		gm.	*gm.*	*gm.*	*Days*		
Basal ('30)	10	308	328	216	17.0	31	41.7
Basal ('32)	9	326	353	229	15.9	36	56.5
Basal plus silage	10	273	323	207	16.8	35	38.8

to death, and average scurvy score for each group (table 1) would indicate that silage has little or no antiscorbutic properties as fed under the conditions of this experiment. While the scurvy score for the silage fed guinea pigs is below that of the basal groups, on the other hand, the average days to first signs of scurvy and the average days to death fall between the values for the basal groups. Ellis, Steenbock and Hart ('21) in earlier studies found silage to contain little if any vitamin C.

In table 2 the results are presented for the controls and the groups of guinea pigs receiving milk from the sources described. The controls which received the basal ration and 2 cc. of orange juice daily, made a more rapid growth with heavier food consumption than any of the milk fed lots.

Six guinea pigs in each lot completed the full 90 days. The others with one exception died at from 49 to 67 days.

Three of the eight animals fed silage milk showed clinical signs of scurvy. This, alone, would constitute evidence in favor of the pasture milk. This evidence is weakened, however, by the fact that only one animal showed clinical symptoms in each of lots III (no silage) and IV (dry ration). If one guinea pig in the silage milk group which died on the twenty-first day were eliminated, there would be no appreciable difference between the different lots in so far as the scurvy score is concerned.

While these results do not indicate any appreciable difference in the antiscorbutic properties of these milks, they quite

TABLE 2

Influence of ration on the vitamin C content of milk

RATION OF COWS	GUINEA PIGS (NO.)	AVERAGE INITIAL WEIGHT	AVERAGE MAXIMUM WEIGHT	AVERAGE FINAL WEIGHT	DRY FOOD CONSUMED	SCURVY SCORE
		gm.	*gm.*	*gm.*	*gm.*	
Controls—no milk	6	313	667	638	2112	0.0
Lot I pasture	9	347	582	555	1529	1.4
Lot II silage	8	327	540	532	1450	7.6
Lot III no silage	8	311	511	478	1472	1.3
Lot IV dry ration for 1 year	10	311	487	458	1173	0.49

obviously produced different effects on growth. It will be observed that the pasture milk group made the most substantial growth followed in order of decreasing growth by the silage, no silage, and dry ration lots. Elvehjem, Hart, Jackson and Weckel ('34) have demonstrated that mineralized pasture milk has a higher nutritive value when measured by rate of gain in rats, which require no vitamin C, than milk from cows on winter feed.

It seems reasonable, therefore, to conclude that the increased growth of the guinea pigs used in the present experiment is not the result of any increased vitamin C values in the milk. This in accord with the scurvy score and clinical findings as well as the results of the chemical analysis.

B. Chemical tests

Since the recently developed chemical tests for vitamin C provide a more satisfactory measure of this substance it was decided to continue the study using a chemical test.

It was possible to obtain, from feeding tests being carried on by the department of dairy husbandry, milk produced by cows on several controlled rations. At the beginning of the experiment ten cows were on the regular winter ration including silage. During the experiment six of these were turned out to young rye pasture. The other ten cows used were on dry winter ration without silage during the whole of this experiment. Evening milk was used for all tests and the titrations were completed within 2 hours after the time the milk was drawn.

As the titrating reagent 2-6 dichlorophenolindophenol was selected because it has been widely used and accepted and because it is claimed to be more nearly specific for vitamin C than other reagents such as methylene blue or ferricyanide mixtures (Tillmans et al., '32; Bessey and King, '33). This reagent, when used with milk serum that had been clarified with trichloracetic acid, was found to give definite reproducible results and to give complete recovery of commercial vitamin C that had been added to samples of milk. This commercial vitamin C was also used to standardize the dye for the other groups. Detailed results for the group of cows that received pasture during the latter part of the experiment are shown in table 3. These results are typical of those found in each group and only the average values are given.

The total numbers of samples tested, the average values of vitamin C in milligrams per liter and the standard deviations are shown in table 4.

The highest individual value observed was 37 mg. and the lowest 17 mg. The highest average for all results on one cow was 29.9 mg. per liter and the lowest similar average was 21.6. It will be seen that the average values for the three different types of milk are nearly identical, with the values for the pasture milk falling between the other two.

The significance of the close agreement between the average values for the different groups is increased by comparison with the large variation between individuals of a group and with the variation of a single individual from day to day. These results confirm the biological findings in this laboratory. The failure of the ration to influence the vitamin C content of milk is in accord with the fact the cow is known to synthesize this vitamin (Thurston, Palmer and Eckles, '29).

TABLE 3

Influence of ration on the vitamin C content of milk (vitamin C expressed as milligrams per liter)

COW NO.	MARCH						APRIL									MAY
	18	20	28	29	30	31	1	6	8	9	15	16	17	29	30	1
1	26	30	24	28	28		30	28	26	21		31	24	26	26	
2	26	30	29	29	29	27	30	31	35	25						
3	26	36	24	28	28	24	28	33	35	26						
4	22	24	19	20	23	23	31	25	29	23						
5	22	27	21	25	23	24	24	26	29	23	29	24	22	26	24	22
6	22	30	25	25	26	24	26	30	29	23	22	26	29	29	24	24
Av.	24	29	24	26	26	25	28	29	30	24	26	27	25	27	25	23

Ration: alfalfa, silage and grain. Pasture after March 28th.

TABLE 4

Influence of the ration on the vitamin C content of milk

TYPE OF RATION	DRY RATION + SILAGE	PASTURE	DRY RATION, NO SILAGE
Number of samples tested	74	57	66
Vitamin C milligrams per liter	25.8	26.5	26.8
Standard deviation	4.0	3.2	3.9

SUMMARY

The influence of a number of carefully controlled rations on the vitamin C ascorbic acid content of milk was determined both by biological assay and chemical test.

Milk from cows receiving pasture was compared with milk from cows in dry lot receiving either silage or dry feed alone.

The results of these experiments indicate that the rations studied have no significant influence on the vitamin C content of milk.

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THE VARIATION IN THE MINERAL CONTENT OF VEGETABLES [1]

JEHIEL DAVIDSON AND J. A. LECLERC
Food Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

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INTRODUCTION

A balanced diet contains the five main types of food components: proteins, carbohydrates, fats, vitamins, and minerals. Deficiency in any of these food constituents causes material disturbances or diseased conditions.

The metabolic disturbances due to protein and vitamin deficiencies have been the subject of much study. Although disturbances due to the inadequate supply of minerals have been studied less than those due to other dietary deficiencies, nevertheless, several such disturbances have been recorded mostly within recent times.

The relation of goiter to an inadequate supply of iodine in the diet has long been recognized. The relation of secondary anaemia to an iron and copper deficiency is now well known (Elvehjem, '33). Functional disturbances due to a deficiency of manganese (Orent and McCollum, '31), and magnesium (Kruse, Orent and McCollum, '32) in the diet have been recorded recently by McCollum and his co-workers.

All these minerals are found in foods in relatively low concentrations or as in the case of magnesium, are required in very minute quantities to prevent functional disturbances. However, rickets exemplifies a nutritional mineral disturbance in which major ash constituents are involved, that is, calcium and phosphorus (McCollum, '22).

[1] Food Research Division contribution no. 230.

Klinke ('31) records abnormal mineral metabolism, including the major ash constituents, in a number of diseases, which are perhaps caused by the improper quantitative relation between the mineral constituents in the diet.

The major ash constituents also play an important role in determining the acid-base balance of foods—a field in animal nutrition which has been little explored since the work of Sherman and Gettler ('12), and Blatherwick ('14) but in which a revived interest is recorded in recent literature (Bell, '33; Kugelmas and King, '33).

Notwithstanding the importance of minerals in nutrition, the available data on the mineral composition of foods are incomplete and unreliable. The data found in Wolff's 'Aschen analysen' (1880) which has been for a long time the chief source of information on the mineral content of plant materials, are now considered unreliable and out of date in view of the cruder methods of analysis used at the time the tables were composed. Ragnar Berg ('25) published with reluctance his compilation of data on this subject, stating that in a general way the data were incorrect and misleading.

Earlier investigators apparently did not realize the significance of the variations in the mineral content of food plants. Their analyses were for the most part made on very few samples and very often on only one sample obtained at random. The object of the present work was to study the variation of the mineral composition of vegetables with variety, climate, soil, season, etc.[2] Leafy vegetables were studied first because of their increasing use in modern nutrition and because theoretically they are more subject to variation than fruits and seeds (Davidson, '33).

[2] When this work had been completed there appeared in Die Landwirtschaftlichen Versuchstationen (vol. 119, 1934, nos. 1–6 and vol. 121, 1934, nos. 5–6) two investigations dealing with the influence of fertilizers, variety and environment on the mineral composition of German forage crops.

EXPERIMENTAL

Materials used. The materials used in this investigation comprised six samples of spinach, six of kale, three of broccoli, two of lettuce and two of cauliflower.[3] In collecting the samples the following information was sought: a) variety of vegetable; b) where grown; c) type of soil; d) fertilizer treatment; e) was land irrigated?; f) analysis of irrigation water; g) kind of insecticide used; h) dates of planting and harvesting.

All these factors should theoretically affect the composition of the vegetables and might suggest an explanation of differences obtained in their analysis. Such information as was obtained from the growers regarding the materials used in this investigation is given in diagram 1.

Preparation of samples for analysis. An entire crate was used as one sample. The sound edible part of the material was weighed as soon as it was obtained, disregarding the weight of the foreign material which in first class vegetables is generally insignificant. In the case of spinach, however, several samples contained an undue amount of sand. In such cases the sand was collected during washing and its weight subtracted from that of the fresh sample.

The samples were washed and rinsed with distilled water and the excess of the adhering water was removed with towels or blotting paper. The material was then dried in a dryer (closed type, with fan running at 1300 to 1400 R.P.M.) at 60°C., re-weighed, ground in an iron mill and kept in Mason jars.

Methods of analysis. The methods used were mostly those of the Association of Official Agricultural Chemists ('30).

Potassium was determined as chloroplatinate; sodium by difference; calcium as oxalate titrated against N/10 potassium permanganate; magnesium gravimetrically; phosphorus volumetrically; sulphur gravimetrically, the materials having been ashed with magnesium nitrate; chlorine volumetrically;

[3] The study of these materials was intended as a part of a more extensive investigation, which, however, has been temporarily discontinued.

DIAGRAM 1

Description of vegetables

VEGETABLE [1]	VARIETY	LOCALITY	TYPE OF SOIL	FERTILIZER TREATMENT [3]	COMPOSITION OF IRRIGATION WATER
				lbs.	*p.p.m.*
Lettuce A	....	Open market[2]	....	....	....
Lettuce B	....	Open market[2]	....	....	....
Spinach A	....	Open market[2]	....	....	....
Spinach B	....	Open market[2]	....	....	....
Spinach C	Virginia Savoy	Diamond Springs, Va., Expt. Station Farm	Norfolk Loam	600 7–8–5 before planting 500 9–6–5 after thinning	Not irrigated
Spinach D	Virginia Savoy	Diamond Springs, Va., Expt. Station Farm	Norfolk Loam	Same as spinach C	Not irrigated
Spinach E	Virginia Savoy	Ocean View, Md.	Sassafras Sandy Loam	500 7–6–5 before planting 500 10–5–4 after thinning 500 $(NH_4)_2SO_4$ when half grown	Not irrigated
Spinach F	Virginia Savoy	Ocean View, Md.	Sassafras Sandy Loam	Same of spinach E	Not irrigated
Kale A	....	Open market[2]	....	....	....
Kale B	Dwarf Blue Curled Scotch	Diamond Springs, Va., Expt. Station Farm	Norfolk Loam	1000 5–8–5 before planting 200 $NaNO_3$ when half grown	Not irrigated
Kale C	Dwarf Blue Curled Scotch	Diamond Springs, Va., Expt. Station Farm	Norfolk Loam	Same as kale B	Not irrigated
Kale D	Dwarf Blue Curled Scotch	Ocean View, Md.	Sassafras Sandy Loam	1500 7–6–5 side dressed	Not irrigated
Kale E	Dwarf Blue Curled Scotch	Ocean View, Md.	Sassafras Sandy Loam	1500 7–6–5 side dressed	Not irrigated
Kale F	Tall Scotch Curled	Davis, Calif.	Yolo Silt Loam	Barnyard manure	Na 59, Ca 31, Mg 62, Cl 13, SO_4 22
Broccoli A	....	Open market[2]	....	....	....
Broccoli B	Italian Green Sprouting	College Park, Md.	Sandy Loam	12 (tons) manure 1000 6–6–5	Analysis not available
Broccoli C	Italian Green Sprouting	College Park, Md.	Sandy Loam	Same as broccoli B	Analysis not available
Cauliflower A	Early March	Salinas, Calif.	Very light gravel	Fertilized	Analysis not available
Cauliflower B	Early March	Salinas, Calif.	Very light gravel	Fertilized	Analysis not available

[1] Parts of vegetables analyzed: lettuce, tight heads (outer leaves and butts removed); spinach and kale, loose leaves, butts removed; broccoli, entire plant, except lower tough portion; cauliflower, flowering portion, butts removed. [2] Washington, D. C.

[3] All applications are expressed in pounds per acre. The figures describing the fertilizers refer, in order, to the percentages of nitrogen

and manganese colorimetrically by the periodate method (Davidson and Capen, '29).

Iron was determined by the thiocyanate method. The aliquots of the hydrochloric acid solutions were evaporated to dryness to render the silica insoluble. Skinner and Peterson ('28) state that this procedure interferes with the development of the thiocyanate color. Under the conditions of this work such interference was not observed. After evaporation to dryness the aliquots were taken up with 10 cc. of a 20 per cent solution of hydrochloric acid, oxidized with potassium permanganate until a permanent pinkish color was obtained and allowed to stand overnight. The next morning the solutions were always found decolorized, and the addition of permanganate was resumed until a permanent pinkish color was again obtained. Ten cubic centimeters of a 20 per cent solution of potassium thiocyanate were then added. The color appeared immediately and resisted fading for several hours. A Schreiner colorimeter was used in comparing the unknown with the standard solutions.[4]

Copper was determined by the Biazzo method (Elvehjem and Lindow, '29). Owing to the relatively high content of phosphorus, the copper was precipitated with hydrogen sulphide in an acid solution as suggested by Elvehjem and Lindow ('29). The precipitate was dissolved in nitric acid, and the solution evaporated nearly to dryness in order to avoid adding too much sodium hydroxide in neutralizing it, as the presence of an excess of sodium nitrate seemed to interfere with the development of the color. There was enough iron in the copper aliquots to develop an intense color with the thiocyanate reagent, but when allowed to stand this color faded and finally disappeared. In some cases the solutions were allowed to stand 2 days before the iron thiocyanate color disappeared. In such cases pyridine had to be added again in order to develop the full green color. When allowed

[4] The iron results, although apparently normal were not included in the tables being open to question because of the fact that the materials were ground in an iron mill.

to stand the solution had to be protected against evaporation as the chloroform used in this method slowly escaped through the supernatant layer of water solution. A Klett colorimeter was used.

The acid-base balance determinations were made according to a method developed in this laboratory which is based on the titration of the ash with corrections for sulphur and chlorine lost during ashing (Davidson and LeClerc, '35).

The moisture in the air-dry samples was determined by drying at 130°C. for 1 hour.

RESULTS

The results calculated to the water-free basis are given in table 1.

Two points are brought out clearly from the analysis of the five vegetables studied:

1. There is appreciable variation in the mineral content of the same vegetable when grown under different conditions.
2. Each vegetable studied has its own range of variation.

Several points of interest are brought out when the results of the analysis are correlated with the description of the samples given in diagram 1.

The spinach samples C, D, E, F belong to the same variety. C and D were taken from different points of a field in Virginia, E and F from different points of a field in Maryland. While there is no appreciable variation between the samples taken at different points of the same field, there is a wide variation between the samples taken from different fields, especially in K, Na, Ca, Cl and Mn. Particularly characteristic is the variation in the potassium and sodium content. The Virginia samples are higher in potassium and lower in sodium than those grown in Maryland. The smaller intake of potassium by the spinach plants from Maryland is compensated by a larger intake of sodium—a phenomenon well known in plant physiology. While the climatic conditions of the two fields are not very dissimilar, the soil types are different. The characteristic differences in the potassium and

ERRATA

In table 1 (p. 61) sulphur figures for spinach C, spinach D, spinach E, spinach F, kale B, kale C, kale D, kale E, broccoli B and broccoli C should be divided by two.

TABLE 1

Variations in mineral content and base balance of vegetables

VEGETABLES	MOISTURE IN FRESH MATERIAL	ON MOISTURE FREE BASIS										
		Ash	K	Na	Ca	Mg	P	S	Cl	Mn	Cu	Base balance
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*cc. N/10 alkali per gram of material*
Lettuce A	...	15.2	5.98	0.55	0.33	0.19	0.59	0.25	2.18	0.0069	.....	6.62
Lettuce B	...	13.2	6.27	...	0.45	0.27	0.98	0.29	1.20	0.0015	.....	....
Spinach A	...	19.1	7.75	0.73	0.69	0.33	1.01	0.66	1.94	0.0241	0.0028	....
Spinach B	...	20.8	6.80	...	0.57	0.58	0.79	0.66	1.19	0.0243	0.0033	13.11
Spinach C	94.0	20.8	7.43	0.70	1.26	0.50	0.96	0.96	0.52	0.0065	0.0073	21.87
Spinach D	95.3	21.4	6.94	0.99	1.49	0.47	0.79	0.95	0.58	0.0070	0.0066	23.32
Spinach E	96.0	20.5	4.14	2.37	1.83	0.34	0.81	0.93	0.66	0.0084	0.0045	20.29
Spinach F	93.7	19.7	4.35	1.62	2.11	0.37	0.92	1.07	0.93	0.0694	0.0043	18.12
Kale A	...	26.1[1]	2.90	0.97	1.37	0.32	0.25	0.64	0.71	0.0075	0.0031	....
Kale B	90.2	17.5	4.11	0.77	2.36	0.29	0.56	2.19	1.02	0.0137	0.0035	14.02
Kale C	92.7	15.4	3.49	0.70	2.07	0.33	0.64	2.27	0.96	0.0066	0.0039	10.72
Kale D	90.8	16.4	3.58	0.83	2.12	0.26	0.78	2.28	1.15	0.0060	0.0031	9.63
Kale E	91.2	15.1	4.37	0.83	1.83	0.24	0.68	2.20	1.20	0.0061	0.0056	8.03
Kale F	90.0	12.8	2.38	1.01	1.51	0.74	0.65	1.05	1.11	0.0011	0.0024	9.45
Broccoli A	...	24.7[1]	3.33	0.82	0.92	0.29	0.70	1.42	0.89	0.0032	.....	....
Broccoli B	87.8	12.6	3.60	0.40	1.48	0.22	0.70	2.44	0.78	0.0025	0.0033	3.79
Broccoli C	88.8	12.9	3.63	0.41	1.57	0.21	0.69	2.30	0.87	0.0021	0.0037	4.80
Cauliflower A	91.0	11.5	3.71	0.64	0.20	0.24	0.82	1.13	0.47	0.0009	0.0045	3.19
Cauliflower B	90.6	10.9	3.45	0.60	0.22	0.25	0.84	1.01	0.50	0.0007	0.0047	2.63

[1] Sample was dried and ground without having been previously washed.

sodium content of the samples from the two fields might be due to differences in soil. However, on consulting diagram 1, it is found that these two fields, besides being located on different soil types, have also received different fertilizer treatment. The Maryland field received an application of ammonium sulphate when the plants were half grown. It has been reported elsewhere (Davidson, '33), that a relatively large application of sodium nitrate, which is physiologically an alkaline fertilizer, caused an increased potassium intake by wheat plants. It is therefore possible that in a similar way ammonium sulphate, which is physiologically an acid fertilizer, caused a decreased potassium intake by the spinach plants.

The kale samples B and C were grown in the same field in the same locality as the spinach samples C and D, and the samples D and E in the same field and in the same locality as the spinach samples E and F. There is less variation in the mineral composition between the kale samples grown on different fields than between the corresponding spinach samples. The peculiar differences in the potassium and sodium content in the four spinach samples is not observed in the corresponding kale samples. This would seem to lend support to the possibility that the differences in the potassium and sodium contents of the spinach samples were caused by differences in fertilizer treatment rather than by differences in soil type.

The failure of the sodium nitrate application to cause an increased potassium content in the kale samples B and C as compared with samples D and E may be explained by the fact that this application was not sufficiently large to produce the effect referred to above (Davidson, '33). Furthermore, the two sets of samples cannot be well compared owing to the fact that the kale samples D and E received a much larger application of nitrogen in the complete fertilizer than B and C and there is no information as to how much of this nitrogen was in the nitrate form.

The kale samples B, C, D, E, differ quite markedly from samples A and F, especially in potassium, calcium and sulphur content.

The kale sample F was grown under irrigation. The irrigation water was appreciably richer in sodium and magnesium than in the other minerals. This sample has a higher sodium and magnesium content than the other five kale samples.

The broccoli samples B and C were taken quite close to each other. They differ but little from each other but differ appreciably from sample A, which was obtained from the open market.

The cauliflowers, taken at different parts of the same field, differ but little in composition.

Kale sample A and broccoli sample A have an exceptionally high ash content while the individual ash constituents are not correspondingly high. These samples having been obtained by another laboratory for a special purpose were dried and ground without having been previously washed, thus the high ash content may have been caused by the adhering soil particles.

In table 2 are given the extremes of variation of the ash, and of the individual ash constituents as well as those of the base balance and moisture content for each of the five vegetables studied. In a special column for each vegetable are given the percentages of extreme variation obtained by dividing the higher extreme by the lower and multiplying the result by 100, i.e., by considering the minimum as 100 per cent.

Spinach and kale varied much more than the other vegetables. However, this may be due to the fact that a larger number of samples of these vegetables than of the others was examined.

The range of variation of the total ash content is relatively small and not correlated with the range of variation of the individual ash constituents. This may be explained by the fact that a low content of one ash constituent is often compensated by a high content of another constituent. Correlation between the ash and its individual constituents is further

TABLE 2

Range of variation in composition of vegetables

CONSTITUENTS	LETTUCE TWO SAMPLES		SPINACH SIX SAMPLES		KALE SIX SAMPLES		BROCCOLI THREE SAMPLES		CAULIFLOWER TWO SAMPLES	
	Per cent	*Per cent variation*	*Per cent*	*Per cent variation*	*Per cent*	*Per cent variation*	*Per cent*	*Per cent variation*	*Per cent*	*Per cent variation*
Ash	13.2 –15.2	115	19.1 –21.4	112	12.8 –17.5	137	12.6 –12.9	102	10.9 –11.5	106
Potassium	5.98 – 6.27	105	4.14 – 7.75	187	2.38 – 4.37	184	3.33 – 3.63	109	3.45 – 3.71	108
Sodium	....	...	0.70 – 2.37	339	0.70 – 1.01	144	0.40 – 0.82	205	0.60 – 0.64	107
Calcium	0.33 – 0.45	136	0.57 – 2.11	370	1.37 – 2.36	172	0.92 – 1.57	171	0.20 – 0.22	110
Magnesium	0.19 – 0.27	142	0.33 – 0.58	176	0.24 – 0.74	308	0.21 – 0.29	133	0.24 – 0.25	104
Phosphorus	0.59 – 0.98	166	0.79 – 1.01	126	0.25 – 0.78	312	0.69 – 0.70	101	0.82 – 0.84	102
Sulphur	0.25 – 0.29	116	0.66 – 1.07	162	0.64 – 2.28	356	1.42 – 2.44	172	1.01 – 1.13	112
Chlorine	1.20 – 2.18	182	0.52 – 1.94	392	0.71 – 1.20	169	0.78 – 0.89	114	0.47 – 0.50	106
Manganese	0.0015– 0.0069	460	0.0065– 0.0694	1068	0.0011– 0.0137	1245	0.0021– 0.0032	152	0.0007– 0.0009	129
Copper	....	...	0.0028– 0.0073	261	0.0024– 0.0056	233	0.0033– 0.0037	112	0.0042– 0.0045	107
Base balance[1]	....	...	13.11 –23.32	178	8.03 –14.02	175	3.79 – 4.80	127	2.63 – 3.19	117
Moisture	....	...	93.7 –96.0	102	90.0 –92.7	103	87.8 –88.8	101	90.6 –91.0	104

[1] Cubic centimeters of N/10 alkali per gram of dry material.

ERRATA

In table 2 of the paper on "The variation in the mineral content of vegetables," by Davidson and LeClerc, this journal, volume 11, number 1, page 64, January, 1936, the sulphur figures should read: for spinach, 0.465–0.66 142; for kale, 0.64–1.14 176; for broccoli, 1.15–1.42 123.

obscured by the fact that two major (referring to quantity) ash constituents, the silica and carbon dioxide, were not determined.

The range of variation of potassium and phosphorus is in general smaller than that of the other major elements, possibly because vegetables are usually grown with complete fertilizer, which always contains these two elements.

The range of variation of the base balances is significant. In the study of the effect of the acid-base balance of foods on animal metabolism a variation of 178 per cent, as in the case of the spinach samples, would preclude any possibility of correlating the acidity and ammonia content of the urine with acid-base balance values found in the literature. For close correlations the acid-base balance values will have to be determined in each case.

The analytical data on the whole bear out the main thesis which it was intended to prove by this investigation, namely, that the mineral content of vegetables must be studied with the object of determining the ranges of variation rather than single values.

SUMMARY

Several samples of each of the vegetables, spinach, kale, lettuce, broccoli and cauliflower were analyzed for total ash, potassium, sodium, calcium, magnesium, phosphorus, chlorine, iron, manganese, copper and the base balance. The conditions under which most of the vegetables were grown are described.

The results show that—

1. There is considerable variation in the mineral content and base balance values of each vegetable studied.
2. Each vegetable studied has its own range of variation.
3. In several instances the mineral composition of the vegetables seems to be correlated with the fertilizer treatment or with the chemical composition of the irrigation water.

The results also seem to lead to the general conclusion that the mineral content of vegetables should be studied with the object of determining ranges of variation rather than fixed values.

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STUDIES ON VITAMIN $G(B_2)$ AND ITS RELATION TO CANINE BLACK TONGUE [1]

C. J. KOEHN, JR. AND C. A. ELVEHJEM
Department of Agricultural Chemistry, University of Wisconsin, Madison [2]

TWO FIGURES

(Received for publication September 16, 1935)

In a previous paper (Elvehjem and Koehn, '35) we reported that the symptoms of pellagra produced in chicks by feeding a heat-treated natural grain ration could not be cured by supplementing the basal ration with flavin preparations from liver or liver extract, but that the pellagra was readily prevented or cured when the filtrate from which the flavin had been separated was used as the supplement. It was suggested therefore that the term vitamin $G(B_2)$ be retained for the factor remaining in the filtrate which cured the pellagra, and that the classification of the flavins be postponed until more was known about their biological activity.

Shortly after the appearance of our work, three independent groups of workers presented data obtained from studies on rats which verified our experimental results. Chick, Copping, and Edgar ('35) found that the addition of hepatoflavin or lactoflavin to a basal diet deprived of vitamin G restored the growth in rats to a small extent but was without effect on the characteristic florid dermatitis. A supplementary substance obtained from yeast extract had no effect in restoring growth but did have a slight curative effect on the dermatitis. When the flavin and supplementary material from yeast were given

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[2] Supported in part by a grant from the research funds of the university.

together, growth and cure of the dermatitis resulted. György ('35 a) found lactoflavin to have no connection with the pellagra-like dermatitis in 211 rats. He also found a combination of the flavin and a supplementary substance necessary for normal performance, and that the distribution of the two substances in nature varies to a considerable extent. Harris ('35) also found flavins to have no antipellagric action. All these workers discuss the possible nomenclature for the factors in question and suggest that vitamin G should be used for the complex and that new names should be given to the several constituents. However, these workers are not in agreement concerning the designation of the individual factors.

The final decision concerning this question must be left to a competent committee, but since the term vitamin G(B_2) has always been associated with the P-P factor of Goldberger, a question of prime importance is the relation of these factors to human pellagra.

At present there is no definite evidence in the literature to demonstrate that the pellagra obtained in chicks is identical with human pellagra. This is also true in the case of rats. Although Goldberger and Lillie ('26) described the production of specific symptoms in rats peculiar to vitamin G low diets which they conclude with some reservation to be the analogue of pellagra in man, the vitamin G low diets used in most laboratories today are not only different from the one described by Goldberger and Lillie, but the typical symptoms are rarely seen in many laboratories.

Eventually the different factors which have been separated must be tested on uncomplicated cases of human pellagra. However, since clinical studies of this nature are difficult to conduct, and since most authorities today agree that black tongue in dogs is identical with human pellagra both from symptomatic and etiological standpoint (Wheeler, '30), we were interested in repeating our work on the separation of vitamin G and flavins when dogs were used as the experimental animal.

The work to be reported in this paper was done on liver extract[3] similar to that used in the experiments with chicks. Goldberger and Sebrell ('30) have shown that liver extract no. 343 given by mouth will cure black tongue in dogs. Rhoads and Miller ('33) prevented black tongue in dogs by feeding 4 gm. daily of liver extract. Ramsdell and Magness ('33) and Ruffin and Smith ('34) have found that large amounts of liver extract by mouth are efficacious in treating human pellagra, while Spies ('34) has demonstrated that either the intravenous or the intramuscular administration of large amounts of potent liver extract causes a remission of the oral manifestations of pellagra.

EXPERIMENTAL

The basal ration was essentially diet no. 323 of Goldberger, Wheeler, Rogers, and Sebrell ('30) except that the cow peas were omitted, and the calcium supplied as calcium carbonate and calcium phosphate. It had the following composition, yellow corn 72, casein (heated 120°C. 24 hours) 18, cotton seed oil 5, cod liver oil 2, calcium carbonate 1, calcium phosphate 1, and NaCl 1, and will be referred to in this paper as ration 250. The dry mixture was prepared fresh each week and for feeding was suspended in hot water, cooked for a few minutes, and fed ad libitum twice daily. Two litters of dogs were used. The dogs in litter no. 1 (Collie breed) were weaned at 4 weeks of age, placed on ration 250 and commercial dog food for 5 weeks and restricted to the basal ration alone at 9 weeks of age. The dogs in litter no. 2 (rat terrier breed) were weaned at 4 weeks of age and started on the basal ration when 6 weeks of age. All dogs but one developed typical black tongue in 4 to 10 weeks. One animal in litter no. 2 for some unknown reason failed to develop the typical symptoms shown by his litter mates. The growth records for two dogs from litter no. 1, which were allowed to die of black tongue are shown in figure 1.

[3] We are indebted to Dr. H. W. Rhodehamel of Eli Lilly and Company, Dr. C. Nielson of the Abbott Laboratories, and Dr. David Klein of the Wilson Laboratories, who kindly supplied us with liver extract.

The symptoms observed were anorexia, loss of weight, lesions and hemorrhage of the tongue and labial and buccal mucous membranes of the mouth, excessive secretions from the eyes, vomiting, bloody diarrhea and general emaciation.

Dogs receiving supplements were placed in separate cages and given the material to be tested in a small amount of the basal ration each morning. After this material was consumed, the basal ration alone was supplied ad libitum. In cases of

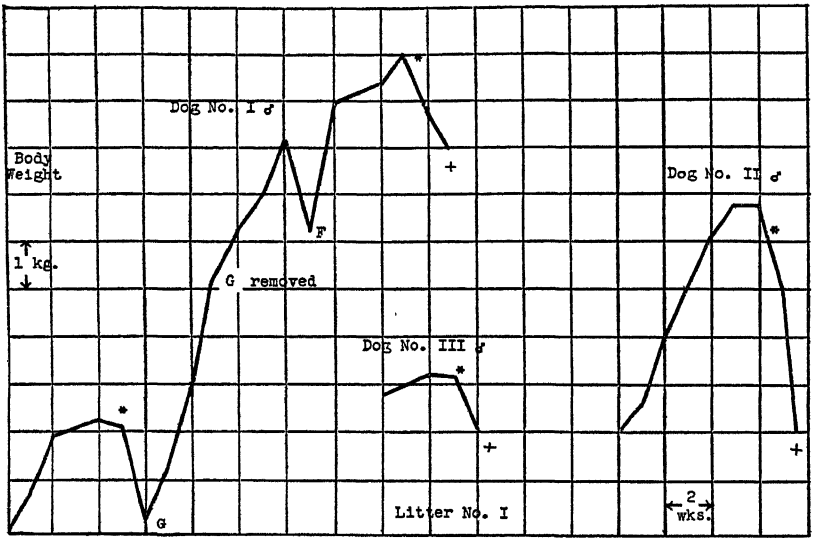

Fig. 1 Growth records of dogs receiving the basal ration and basal ration plus supplement. G denotes the administration of the vitamin G supplement. F denotes the administration of the flavin supplement. * appearance of black tongue.

severe black tongue, where the dog completely refused to eat, the supplement was given orally by pipette.

The liver extract was separated into the two fractions in the following manner: 100-gm. portions of liver extract were dissolved in 200 cc. of distilled water, and 1 liter of ethyl alcohol and 1.2 liters of diethyl ether were added with constant stirring. The solution was filtered, the precipitate washed with 250 cc. of a mixture of alcohol, ether, and water, and the washings combined with the filtrate. The solution was concentrated in vacuo to a volume of approximately 50 cc., filtered

and diluted to 1 liter with distilled water. Sixty cubic centimeters of concentrated HCl and 35 gm. of fuller's earth were added and the mixture shaken for 1 hour in a mechanical shaker. The fuller's earth was allowed to settle out over night, the supernatant liquid siphoned off, and the earth drained on quantitative filter paper. It was then washed by suspending in two 1-liter portions of distilled water.

The flavin was eluted by suspending the fuller's earth in a mixture of 250 cc. of water, 65 cc. of pyridine, and 65 cc. of methyl alcohol, and shaking for 1 hour. The fuller's earth was removed by centrifuging, the liquid concentrated in vacuo to 10 cc. to remove the pyridine, methyl alcohol, and colloidal fuller's earth, and diluted to 200 cc. In the above procedure, the operations were carried out in such a manner as to exclude exposure of the solutions to light.

The filtrate remaining after the flavins had been removed with fuller's earth was neutralized with NaOH and concentrated in vacuo to 50 cc. Three hundred cubic centimeters of 95 per cent ethyl alcohol were added and the precipitated NaCl was filtered off. The solution was concentrated to 10 cc. and diluted with water to 100 cc.

These supplements were fed at a level equivalent to 5 gm. of liver extract daily. This level was found by Goldberger and Sebrell ('30) to be just sufficient to prevent black tongue.

Dog no. 1 of litter no. 1 stopped growing at the end of the fourth week and developed severe black tongue at the end of the fifth week. At the end of the sixth week the vitamin G fraction was administered whereupon the symptoms disappeared and a rapid gain in weight followed. This supplement was given for 3 weeks and then removed. The dog continued growing for 3 weeks and then declined in weight. The flavin fraction was then used to supplement the ration. For 3 weeks the dog gained weight, then developed severe black tongue and died within 2 weeks (fig. 1).

In litter no. 2, dog no. 4 began losing weight at the end of the eighth week. The flavin fraction was administered whereupon severe black tongue developed with a rapid loss in

weight for 3½ weeks. The flavin supplement was then replaced by the vitamin G fraction. A very rapid increase in weight occurred with immediate disappearance of black tongue symptoms (fig. 2). After receiving this supplement for 5 weeks, the dog was killed and autopsied. Autopsy showed all organs to be normal. There were indications that there had been lesions in the lining of the small intestines but these lesions had been completely healed at the time of autopsy.

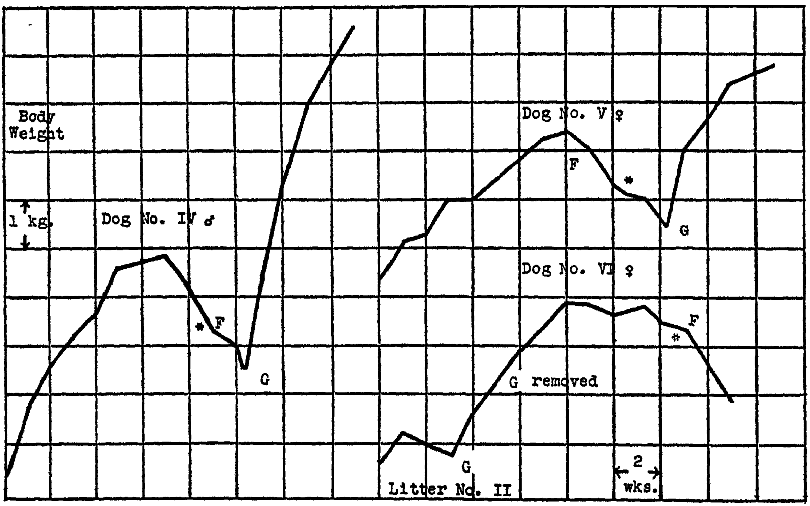

Fig. 2 Growth records of dogs receiving basal ration plus flavin fraction or vitamin G fraction. G denotes the administration of the vitamin G supplement. F denotes the administration of the flavin supplement. * appearance of black tongue.

Dog no. 5 stopped growing at the end of the eighth week and started losing weight. The ration was supplemented by the flavin fraction whereupon black tongue developed and the loss of weight continued. After receiving the flavin supplement for three weeks, the dog exhibited very severe symptoms of black tongue and extreme emaciation. The flavin fraction was withdrawn and replaced by the vitamin G fraction. Immediate disappearance of the symptoms of black tongue and resumption of growth occurred (fig. 2). The dog was killed and autopsied after receiving the vitamin G fraction for 5 weeks.

Autopsy as before revealed healed lesions of the intestinal tract.

Dog no. 6 lost weight after the end of the first week. At the end of the third week the vitamin G fraction was administered. Immediate resumption of grow resulted. After 3 weeks the supplement was removed and the dog continued growing for several weeks. At 13 weeks black tongue developed and the flavin supplement was given. Rapid loss in weight and extreme emaciation resulted. Autopsy revealed severe lesions and hemorrhages in the gastro-intestinal tract.

DISCUSSION

The fact that flavin prepared from liver extract has proven ineffective in the cure of canine black tongue, which has been shown to be analogous to human pellagra, gives additional and more conclusive evidence that flavins are not the antipellagric vitamin. Further support is given to the suggestion that vitamin G be retained for the flavin-free fraction active in the prevention of pellagra. Whether the fraction which has been found active for both chicks and dogs contains only one specific active substance cannot be answered at this time. Our work on the purification of the antipellagric factor indicates that there is only one substance concerned with this deficiency disease.

It is also difficult to determine whether the factor we have been concerned with is identical with the active principle studied by Chick ('35), György ('35 a), and Harris ('35), which they found in a yeast preparation. György suggested that the pellagra preventing substance in yeast as tested on rats be designated vitamin B_6. In this connection it is well to mention that György ('35 b) found whole liver to be a rich source of this factor. However, in a later publication Birch and György ('35) state that from a study of the distribution of B_6 and the flavin in cereal products, it appears highly probable that neither of these substances is identical with the human P-P factor of Goldberger. György ('35 c) also found the pellagra-preventing substance as studied in rats (vitamin

B_6) to be destroyed by visible light. We have been unable to demonstrate any decrease in the potency of concentrates active in the prevention of pellagra in chicks when exposed to either visible or ultra violet light. Reference is made to the work of Hogan and Richardson ('34) who showed that the pellagra-like dermatitis in rats was produced more readily when certain components of the diet were irradiated. György repeated this work and concluded therefrom that the supplementary factor (vitamin B_6) will cure the dermatitis produced by Hogan's method. However, in a recent paper Hogan and Richardson ('35) conclude that the dermatitis in their rats is cured by an ether extract of wheat germ. Thus there are probably several types of dermatitis and several factors concerned in the prevention of the development of each of these syndromes. Very recently von Euler and Malmberg ('35) report definite skin changes in rats on their basal diet plus 10 γ flavin and a flavin-free yeast preparation. Highly purified rations of casein, sugar, or starch and salts must be deficient in a number of factors, and different symptoms are encountered depending upon the purification of the casein and starch and the nature of the supplements used as sources of the known vitamins. If we are to test for the true antipellagric vitamin, all factors but the one in question must be present. This means that flavins must be present in the basal diet as well as vitamins A, D, B, etc. and not included as part of the vitamin G complex. If we accept the terminology of the committee on vitamin B nomenclature of the American Society of Biological Chemistry ('29) that G(B_2) be used to denote the more heat-stable, water-soluble, dietary factor called the P-P factor by Goldberger and associates, and which also has to do with maintenance and growth, then it seems most logical to call the factor which remains after the flavins are removed on fuller's earth and which has been shown to be active in the prevention of pellagra-like symptoms in chicks and black tongue in dogs, vitamin G(B_2).

SUMMARY

1. Typical symptoms of black tongue were produced in dogs by feeding a modified Goldberger ration.

2. Preparations of hepatoflavin prepared from liver extract were found to be completely inactive in the prevention or cure of black tongue.

3. Fractions prepared from liver extract which were rich in vitamin G(B_2) and from which the hepatoflavin had been removed through adsorption on fuller's earth were very active in the cure of black tongue.

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EFFECTS OF EXCESSIVE INGESTION OF SODIUM AND POTASSIUM SALTS ON CARBOHYDRATE METABOLISM AND BLOOD PRESSURE IN DIABETIC CHILDREN [1]

IRVINE McQUARRIE, WILLIS H. THOMPSON AND JOHN A. ANDERSON
Department of Pediatrics, University of Minnesota, Minneapolis

NINE FIGURES

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In a preliminary communication (Thompson and McQuarrie, '34), the finding of an apparently significant influence of excessive salt ingestion on arterial blood pressure and carbohydrate metabolism was reported. The present paper records the results from a more extensive investigation of the subject.

Our interest in the question of a possible interrelationship between salt ingestion and carbohydrate metabolism was first aroused in 1930, when one of our younger diabetic patients, suffering from an abnormal craving for salt, appeared to show some freakish variations in her ability to utilize carbohydrate according to variations in the amounts of sodium chloride which she was allowed to consume. Lack of cooperation on the part of the patient and her premature discharge from the hospital frustrated our first attempts to investigate the problem. A second opportunity presented itself, however, in the autumn of 1933, when one of our clinic patients, a 15-year-old boy with moderately severe diabetes manifested a similar but even more marked craving for salt.

Obviously the first question presenting itself was that regarding the factors responsible for this patient's requiring

[1] Aided by a grant from the Medical Graduate Research Fund of the University of Minnesota.

between 80 and 90 gm. of table salt daily to satisfy his abnormal craving. Since he had been on a relatively high vegetable diet (high in K) for a long time, had partially depleted his reserve of base as a result of ketosis and had vomited an undetermined number of times just before being admitted to the hospital (thereby losing a considerable amount of chloride), this symptom was first tentatively explained on the simple basis of temporarily physiological need for both Na and Cl. That this explanation was entirely inadequate soon became apparent, however, when the craving was observed to continue only slightly abated long after all evidence of ketosis had disappeared and after a simplified diet low in potassium had been substituted for the ordinary diabetic diet. The unexpected discovery, that this patient's blood pressure was elevated temporarily and his glycosuria was decreased as a result of the extra salt ingestion, impressed us as being of sufficient interest to warrant immediate investigation. It appeared probable too that the factors involved in these reactions might incidentally provide an answer to our query concerning the symptom of salt craving.

Following confirmation of our original observation regarding the effects of high NaCl intake, a number of pertinent new questions presented themselves. Among these were the following: 1) Are the observed reactions peculiar to diabetic subjects suffering from an abnormal craving for salt or do other diabetics and even normal persons behave similarly under the same conditions? 2) Are the observed effects due specifically to the Na or the Cl ion alone or to the two combined? 3) Are the two effects (i.e., decrease in glycosuria and increase in blood pressure) mutually dependent or is it possible to obtain one without the other? 4) May the effect on blood pressure be due to increased tonus in the peripheral arterioles, to effects on the kidneys or merely to the mechanical effect of increased blood volume? 5) Is the diminution in glycosuria due merely to retention of sugar with retained water or is it due to improved ability to store or oxidize glucose? 6) If either of the latter, what is the nature of the

mechanism involved? 7) What nervous or endocrine functions, if any, are involved? 8) Has a high salt intake any influence on the metabolism of protein and fat?

METHODS AND MATERIALS

Repeated studies were carried out on each of four diabetic patients and on one normal subject, all in the age group between 13 and 15 years. Not more than two patients were under observation at one time. At such time they were kept in a small metabolism ward constantly under the supervision of special nurses, who weighed them every 12 hours, collected the urine and feces in special containers and made certain that diets and medications were taken precisely as ordered. Urine was collected quantitatively in 6- or 12-hour periods. The feces for different periods were marked by carmine according to standard technic. Systolic and diastolic blood pressure measurements were made by means of the same mercury sphygmomanometer before meals and after the patient had been in the recumbent position for at least ½ hour. Measurements were frequently checked independently by two observers. The averages of these readings were charted each day. The room temperature was held between 68° and 74°F. and the relative humidity between 40 and 60 per cent to prevent sweating or chilling. The body weight was determined every 12 or 24 hours immediately after the bladder was emptied and before meals or water were given.

The daily diet, which was constant for each patient throughout the entire experimental period, was divided into four identical meals given at 6-hour intervals. For the sake of uniformity and accuracy, it was made up from the following foods: powdered whole milk, egg yolk, egg white, cane sugar, water, unsalted butter, clear lemon juice and a small amount of white bread. The mineral constituents of a typical basic diet, containing protein 64, fat 96, and carbohydrate 132 gm., were as follows; Na, 1.03; K, 1.34; Ca, 0.68; Mg, 0.16; Cl, 1.83; P, 0.93 and S, 0.76 gm. It is seen that this is relatively low in sodium, potassium and choride. The amounts of protein,

fat and carbohydrate were adjusted to meet each patient's caloric and nitrogen requirements. The total water intake, based on the maximum amount taken during periods of high NaCl ingestion, was the same throughout individual experiments. The insulin dosages, in those cases on insulin therapy, were given at 6-hour intervals and were adjusted at levels which allowed some degree of glycosuria in every period of the day. When extra salt was added, it was given in the form of weak solutions and in gelatin capsules, the total for the day being administered along with the diet in four equal parts. Only chemically pure salts were used.

The urinary glucose was determined on the fresh samples in 6- or 12-hour periods by the method of Benedict ('11). The total nitrogen and the various mineral constituents were determined on 24-hour samples. The chemical methods used for the analyses of urine and feces were as follows: total nitrogen, Kjeldahl (1883); sodium, Salit ('32); potassium, Shohl and Bennett ('28); chloride, Volhardt, as modified by Harvey ('10). For blood analyses the methods were as follows: glucose, Folin and Wu ('20); sodium, Butler and Tuthill ('31); potassium, Shohl and Bennett ('28); chloride, Wilson and Ball ('28). The blood volume was determined by the dye method of Keith, Rowntree and Geraghty ('15) as modified by Hooper, Smith, Belt and Whipple ('20). Fasting respiratory quotients were kindly determined for us by Mr. A. J. Beber in the laboratory of Dr. George Burr.

EXPERIMENTAL RESULTS

For the sake of brevity, representative data from the various experiments are here presented by means of one short table and a series of graphic charts.

Table 1 contains the data from a single study on the various effects produced by the ingestion of 60 gm. of NaCl daily for a period of 1 week in the case of L.R., the 15-year-old boy who craved salt. It will be seen that the degree of glycosuria fell far below the control level soon after the extra salt was introduced and remained comparatively low throughout the experi-

mental period. That this change was due to the salt is further evidenced by the fact that glucose output again gradually rose to its previous level during the 4-day-post-saline control period. Approximately 27 gm. of NaCl was retained during the first day of the experimental period, but thereafter the output was practically equal to the intake. The gain of 2.5 kg. in body weight, which accompanied the retention of salt, undoubtedly represented chiefly retention of water. At an assumed Na concentration of 152 milli-equivalents per liter, that

TABLE 1

The effects of ingesting 60 gm. of NaCl daily on L.R. age 15 years. Severe diabetic. Diet contained protein 64, fat 132 and carbohydrate 168 gm. Insulin 32 units daily. No ketosis

DAY	INTAKE (GRAMS)				OUTPUT (GRAMS)					WEIGHT (KILOGRAMS)	BLOOD PRESSURE
	Na	Cl	K	N	Na	Cl	K	N	Glucose		
1	1.57	2.43	1.48	10.36	1.86	2.27	0.86	12.40	73	38.3	116/80
2	25.16	38.84	1.48	10.36	14.26	13.38	0.86	11.10	33	38.2	116/76
3	25.16	38.84	1.48	10.36	24.17	42.28	0.86	7.87	13	40.8	140/88
4	25.16	38.84	1.48	10.36	23.55	38.16	1.40	9.23	14	40.3	150/98
5	25.16	38.84	1.48	10.36	26.56	43.06	0.93	9.63	33	40.3	174/110
6	25.16	38.84	1.48	10.36	24.38	33.58	1.56	9.67	31	40.2	166/108
7	25.16	38.84	1.48	10.36	24.83	40.36	1.40	9.78	26	40.4	164/112
8	25.16	38.84	1.48	10.36	27.28	41.85	1.21	10.87	26	40.4	176/118
9	1.57	2.43	1.48	10.36	4.99	7.88	1.44	8.78	30	40.4	164/98
10	1.57	2.43	1.48	10.36	1.86	2.41	0.82	8.91	49	39.2	130/88
11	1.57	2.43	1.48	10.36	2.57	3.80	1.13	9.47	57	39.1	112/80
12	1.57	2.43	1.48	10.36	1.75	2.24	1.05	10.87	72	38.7	106/74

ordinarily assigned to extracellular body water, the retained water would account for but 22 gm. of the NaCl not excreted by way of the urine and feces, leaving 5 gm. unaccounted for. Since visible sweating did not occur, it is obvious that the concentration of NaCl in the body fluids was increased or that the extra salt was stored in some other form than in aqueous solution. Actual determinations of the Na and Cl in the blood serum a few days before and again a few days after the ingestion of the extra 60 gm. of NaCl per day were as follows: before, Na 303 mg. and Cl 281 mg. per 100 cc. of serum; after:

Na 342 mg. and Cl 342 mg. per 100 cc. The fasting blood sugar values for the corresponding days were 290 and 131 mg. per 100 cc. respectively. Increased concentration of salt in the body fluids of this particular patient, therefore, account in all probability for most of the extra NaCl retained. A comparison of the average per diem excretion of potassium for the experimental period with that for the fore-period and after-period shows an increased output of this element during the period of high NaCl intake.

The total nitrogen excretion was definitely decreased during the periods of high salt ingestion. As may be observed from table 1, the nitrogen balance was negative during the low sodium chloride period when glycosuria was most marked, but was positive during the high salt period. This protein-sparing effect has been observed repeatedly in the series of experiments thus far completed. It is an additional proof of improved carbohydrate utilization.

The systolic and diastolic blood pressure levels both rose well above the upper limits of normal after the second day of high salt ingestion and remained elevated until the second day following withdrawal of the salt. The systolic level was increased approximately 40 per cent while the diastolic rose about 30 per cent. All of the changes recorded showed a period of lag at the beginning and at the end of the experiment, accompanying respectively the initial retention and then the gradual excretion of extra salt. The patient complained of headache on several occasions when his systolic pressure rose above 165 mm. of mercury.

Certain of the data from the foregoing experiment are presented graphically in figure 1, which is largely self-explanatory. In addition, the chart shows the ineffectiveness of adrenal cortical extract (Eschatin P.D.) as a substitute for NaCl in this relationship, when given in average-sized doses. Obviously this is not a crucial or altogether satisfactory test of this point because maximum doses were not given.

Since it at first appeared possible that the response to high sodium chloride ingestion just described might be peculiar to

patients suffering from abnormal salt craving, similar studies were made on other experimental subjects both diabetic and non-diabetic, who manifested little or no such craving.

Data are graphically presented in figure 2, which demonstrate clearly that other diabetic subjects than those marked

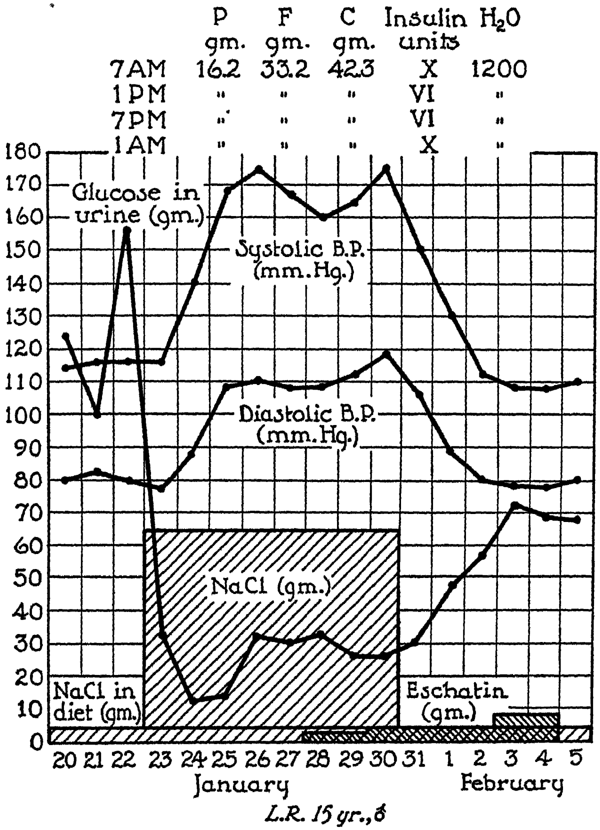

Fig. 1 Effects of sodium chloride on glycosuria and blood pressure. Non-effect of extract of adrenal cortex. L.R. severe diabetes.

by excessive salt craving respond to a sustained high intake of NaCl. It will be seen that the amount of glucose excreted per day by patient H.E. during the fore-period, namely between 40 and 70 gm. fell to between 10 and 20 gm. daily when the NaCl intake was increased from 4 to 48 gm. per day. At

the same time the systolic blood pressure rose from a basic level varying between 108 and 116 mm. of mercury to a new level ranging between 140 and 155 mm., while the diastolic level rose from between 74 and 85 to between 100 and 110 mm. of mercury. Soon after the extra sodium chloride was with-

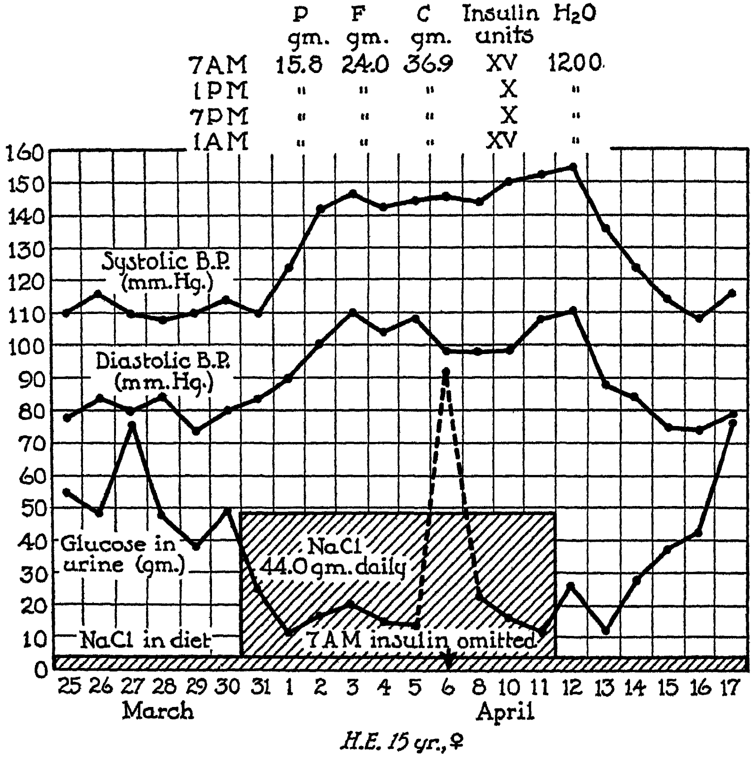

Fig. 2 Effects of sodium chloride ingestion on glycosuria and blood pressure. Dotted line shows effect on glycosuria of omitting insulin for 12 hours. H.E. severe diabetes.

drawn, the previous levels of glycosuria and of blood pressure were resumed.

An additional point of interest is shown in this figure, namely, that withholding of insulin for 12 hours from this moderately severe diabetic subject caused a prompt and marked increase in the degree of glycosuria without a corre-

sponding fall in the blood pressure. The effect of this withdrawal on the glycosuria curve is represented by the interrupted line.

To determine whether or not other than diabetic subjects react to overfeeding of NaCl in the foregoing manner, a single control study was made on a healthy, 14-year-old boy, L.K., who at the time of the experiment was in the late stages

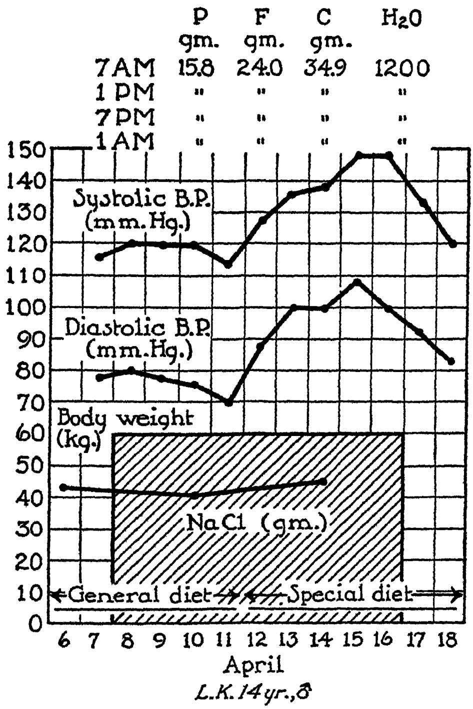

Fig. 3 Response of blood pressure to high-sodium, low-potassium intake in normal subject, L.K.

of convalescence from an operation for club foot. Since he did not have glycosuria or any disturbance of his carbohydrate metabolism, this phase of the problem could not be investigated by means of the technic employed here. It may simply be noted that he did not show any signs of hypoglycemia at the height of the salt effect. The effect on blood pressure, however, was very definitely like that of the diabetic patients.

Reference to figure 3 shows that he had no rise in either his systolic or diastolic pressure from the ingestion of 60 gm. of NaCl daily during the first 5 days while he was on the ordinary hospital diet. However, when he was given the same diet as that taken by patient H.E., namely, the simplified diet, which

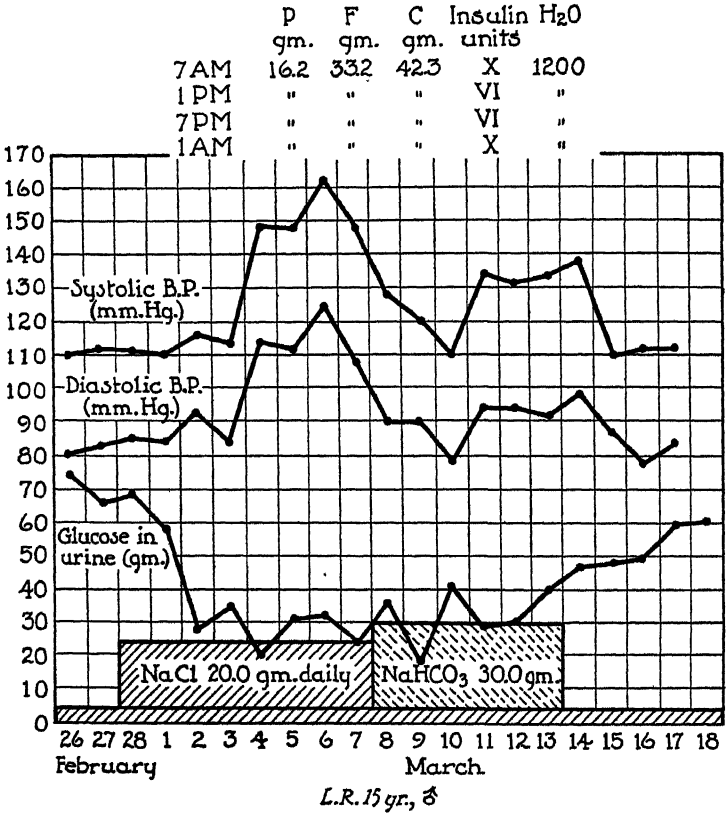

Fig. 4 Effects of sodium bicarbonate on glycosuria and blood pressure. L.R. severe diabetes.

was comparatively very low in K, his systolic pressure rose to between 130 and 150 mm. of mercury, while his diastolic level rose to between 90 and 108 mm. of mercury. These values gradually returned to normal following withdrawal of the extra salt.

The next objective was to determine which of the two ions, Na^+ or Cl^-, is primarily responsible for the reactions observed. The following four charts present data pertaining to this phase of the study.

An attempt to evaluate the specific effect of Na is represented by the data graphically recorded on figure 4. After eliciting the typical reaction to sodium chloride in our most

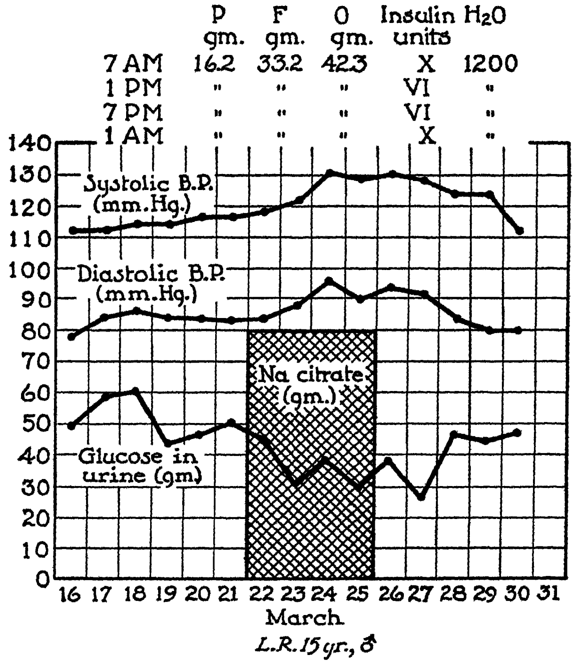

Fig. 5 Effects of sodium citrate on glycosuria and blood pressure.

responsive subject, L.R. (who manifested a craving for salt) with an extra salt intake of but 20 gm. daily, an equivalent amount of Na was substituted in the form of sodium bicarbonate. The object in giving a smaller amount of salt in this experiment than that used in the previous tests was that of avoiding, if possible, a drastic displacement of the acid-base equilibrium of the body fluids toward the alkaline side, which in itself may influence carbohydrate metabolism (Murlin and

Kramer, '16). It is seen that 20 gm. of extra NaCl daily finally resulted in a moderate decrease in the degree of glycosuria and an accompanying elevation of blood pressure after a certain amount of the salt had been retained. When the sodium bicarbonate was substituted, the effects produced by the NaCl were sustained but somewhat less efficiently. Withdrawal of the bicarbonate then resulted in a return of the values for the glycosuria and the blood pressure readings toward their original control levels.

In the next experiment (fig. 5) another sodium salt, the citrate, was administered in daily amounts approximately equivalent to 40 gm. of NaCl. It is evident from the course of the urinary sugar curve and that of the curves representing changes in the blood pressure that the effects of this salt are similar to those of NaCl though less marked. In agreement with the preceding, this experiment appears to indicate that sodium is the element which is responsible in large part for the effects observed.

The next step was to determine the effect of the chloride ion when given with some basic ion other than sodium. The potassium salt was employed because it occurs naturally in the animal body and because, like NaCl, its effects on the acid-base equilibrium are minimal. This salt was found to exert somewhat surprising effects as shown in the two following figures.

Inspection of figure 6 reveals the fact that KCl, when ingested in much smaller amounts, had effects on both the blood pressure and glycosuria of a third diabetic child, J. P., which were diametrically opposite those of NaCl. During the first 4 days shown on the figure, when she was receiving 44 gm. of extra NaCl daily, the urinary glucose varied between 20 and 30 gm. per day and the blood pressure varied between 132/98 and 150/110. Shortly after the extra NaCl was omitted, the glucose excretion rose to between 60 and 70 gm. per day, while the blood pressure fell to a level averaging around 110/78, where it remained throughout the next week. Potassium chloride was then given in gradually increasing doses over the

next 6 days, reaching a maximum of 20 gm. per day for the last 2 days. Apparently as a direct result of this, the glucose excretion increased to new levels varying from 70 to 100 gm. daily, whereas the blood pressure fell slightly below the basic level to between 86/60 and 95/62. Discontinuance of the KCl was followed within 36 hours by a return of all values to the control levels.

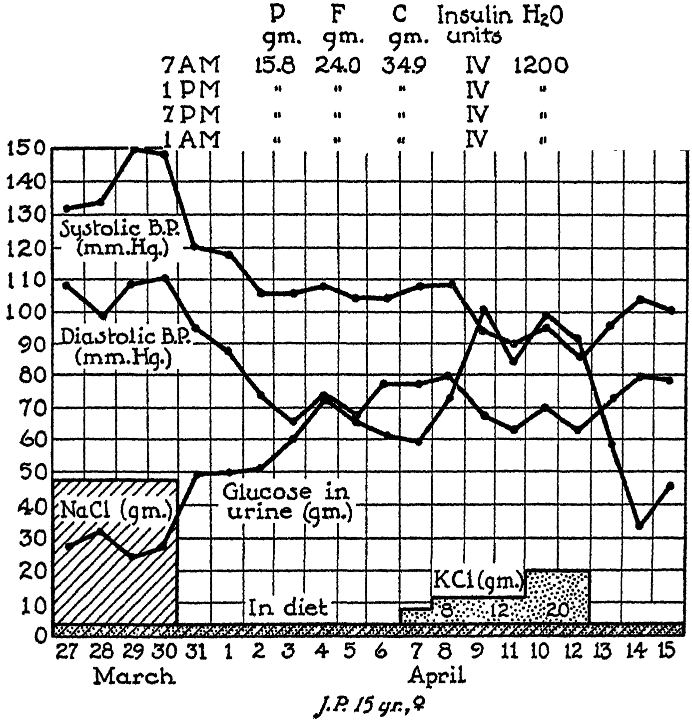

Fig. 6 Comparison of effects of potassium chloride with those of sodium chloride on blood pressure and glycosuria. J. P. moderately severe diabetes.

It was next attempted to ascertain whether or not Na and K exert antagonistic effects when given at the same time and, if so, in what equivalent proportions the one is capable of neutralizing the influence of the other.

Under basic conditions similar to those already described, the patient H.E. was given 40 gm. of NaCl daily over a period of 19 days. Without any other modification of the regimen,

KCl was given as follows: 12 gm. on the eleventh day, 16 gm. on the twelfth and thirteenth days and 4 gm. on the fourteenth day, after which it was withdrawn. The effects of the latter procedure are shown graphically in figure 7. It is seen that 16 gm. of KCl more than neutralized the effects of 40 gm. of NaCl. Since the potassium in 16 gm. of KCl is chemically equivalent to the sodium in but 12.6 gm. of NaCl, it would appear from the present experiment that 1 milli-equivalent of K

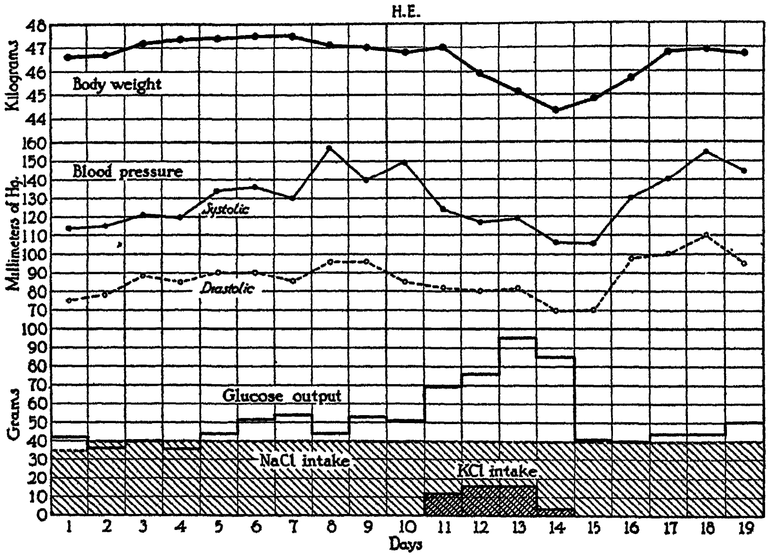

Fig. 7 Antagonistic effects of sodium and potassium on glycosuria and blood pressure. H. E. severe diabetes.

is capable of antagonizing the effect of at least 3 milli-equivalents of Na, as far as the carbohydrate metabolism and blood pressure are concerned. The difference in the effects of the two salts on the water balance of the body should be noted. It is obvious that the sodium ion is chiefly responsible for the retention of water as it is for the decrease in glycosuria and the increase in blood pressure. The decrease in glucose output as a result of NaCl ingestion was far less striking in this than in the previous experiment on H.E. (fig. 2), apparently because she was suffering from a mild upper respiratory infection during the first week of the present experiment.

The studies presented thus far, with the exception of that on one normal subject, were all carried out on severely diabetic patients who required insulin therapy. In order to determine the specific effects of the various salts without this complicating factor, the next experiment was carried out on D.R., a 14-year-old girl, whose diabetic state could be controlled fairly satisfactorily by dietary measures alone. After a pre-

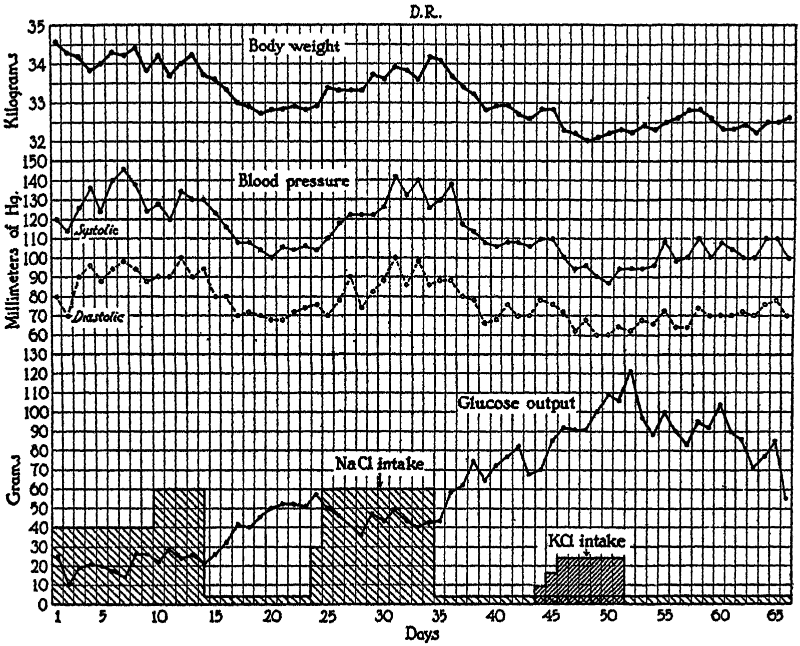

Fig. 8 Effects of sodium chloride and of potassium chloride on glycosuria and blood pressure in diabetic patient not on insulin therapy.

liminary test period, her diet was adjusted at such a level that between 50 and 75 gm. of glucose appeared in the urine daily during control periods. She was then given the salts as indicated in figure 8.

The data recorded on this figure demonstrate that the antagonistic effects of large amounts of NaCl and of KCl were qualitatively the same in this diabetic patient, who was not under the insulin therapy, as they were in the preceding cases.

The fasting serum potassium in the case of this patient was significantly lower during the period of high NaCl intake than during the control periods, the values being 27.2 mg. per 100 cc. before, 12.5 mg. during and 19.6 mg. 1 week after the high-salt period.

The next project undertaken was that of determining the effect of NaCl ingestion on the level of the blood sugar. A

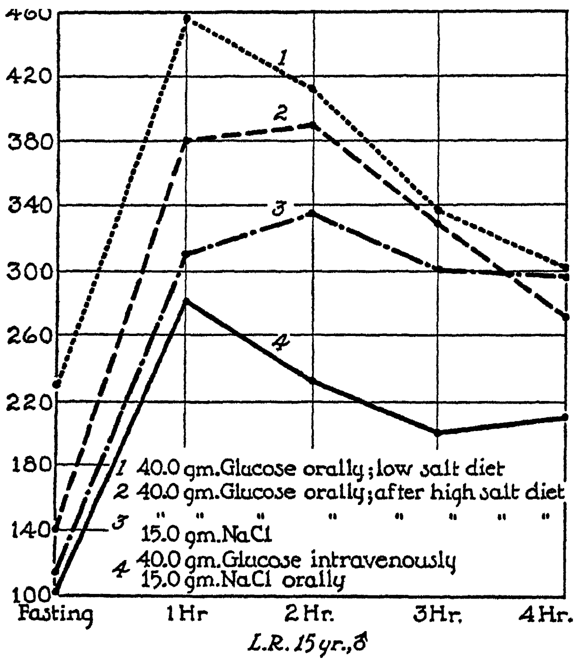

Fig. 9 Effects of sodium chloride on fasting blood sugar and on sugar tolerance curves.

series of sugar tolerance tests were made on patient L.R. under a variety of conditions as indicated on figure 9. It will be observed that all curves obtained were of the diabetic type, the ingestion of salt having no demonstrable beneficial effect under the emergency conditions of such a test. The only difference in the blood sugar findings during the control period and during the periods of high salt feeding was a tendency for the fasting values to be distinctly lower

under the latter circumstances. Nevertheless, this latter finding indicates a definite increase in the ability to utilize carbohydrates.

Additional evidence that the metabolism of carbohydrate is significantly improved by the excessive ingestion of NaCl is found in the fact that the fasting respiratory quotient was found to be slightly higher during the period of high intake than during the period of low intake in the few instances in which this factor was determined. For example, patient H.E. showed an R.Q. of 0.763 on the high salt regimen and 0.714 on the low salt, while corresponding values for L.R. were 0.747 and 0.712. Ketosis was found to develop earlier in the severely diabetic subjects after withdrawal of insulin during the period of low NaCl intake than during the periods of high intake. On several occasions patients, whose insulin dosages were sufficiently large to prevent marked hyperglycemia but not sufficient to cause hypoglycemic reactions during periods of very low NaCl intake, were found to experience typical insulin reactions when from 40 to 60 gm. of this salt were given daily.

Whether or not simultaneous administration of the potassium will prevent the latter reactions has not as yet been determined. In one extremely severe adult case of spontaneous hypoglycemia associated with acromegaly, however, we observed a steady but temporary rise in the blood sugar with partial relief of symptoms following the administration of between 24 and 48 gm. of KCl daily. Much to our surprise, no toxic symptoms were observed in the case of this man, although he finally refused to take the larger doses because of gastric discomfort. In less severe cases of spontaneous hypoglycemia, a low-sodium, high-potassium regimen might prove to be beneficial.

DISCUSSION

The foregoing data afford ample proof that the daily ingestion of sodium chloride in large amounts causes an elevation in the blood pressure and at the same time increases the ability of the diabetic subject to utilize carbohydrate. These effects are not observed if the diet is high in potassium. The effect of potassium chloride was to lower blood pressure and increase glycosuria.

A satisfactory explanation for these effects is not apparent at the present time. The effects referred to are not obtained with the small amounts of salt and water required to prevent depletion of the body's reserves. The reports of Atchley and others ('33) and Peters, Kydd, Eisenman and Hald ('33) and their co-workers have adequately described the disturbances in water and electrolyte balances which develop in uncontrolled diabetes mellitus. Kydd ('33) has emphasized the depletion of chloride in excess of other electrolytes, as if the base lost with the ketone acids might be derived from NaCl rather than from bicarbonate. He stresses the need for replacement of chloride during recovery from diabetic coma. However, in the present studies the dietary and other control factors were purposely adjusted at levels such that ketosis was extremely slight if present at all.

Since the fluid balance of the body is modified to a considerable degree and in opposite direction by the ingestion of large amounts of sodium and potassium salts, this factor must be taken into account. The decrease in glycosuria due to NaCl ingestion followed the retention of water, whereas the increase in glycosuria from potassium administration was accompanied by a mild diuresis. This observation is in keeping with the finding of Schiff and Choremis ('26) that a normal or increased hydration in the tissues favors carbohydrate utilization. It is contrary to the view of Andrews ('26) that dehydration enhances insulin action. That the glucose retained is not merely held in solution in the retained water is easily shown by comparing the small quantity so held with the total amount seen to be retained over the period of a week

(table 1). Assuming the concentration of glucose in the retained water to equal that of the blood plasma (e.g., 150 mg. per 100 cc.), the $2\frac{1}{2}$ kg. of retained water would contain less than 4 gm., whereas in the course of 1 week over 300 gm. were actually retained. In those cases in which insulin therapy was employed, it might be assumed that the beneficial effect of water and salt retention in the extracellular spaces is due to the slower release of subcuaneously injected insulin. This explanation could not hold, however, in diabetics not on insulin therapy. Various data are presented which indicate that glucose is more efficiently oxidized and probably more readily stored as glycogen when large amounts of NaCl are ingested. The protein-sparing effect, fasting respiratory quotient measurements and lessened tendency to development of ketosis, as well as the decrease in glycosuria and hyperglycemia, all point in this direction.

Reference to the literature of the pre-insulin era reveals the fact that other observers obtained similar results from feeding large amounts of sodium salts. For example, Murlin and Kramer ('16) found that sodium hydroxide and carbonate increased in utilization of glucose in diabetic dogs while Murlin and Craver ('16) and Underhill ('17) observed marked decreases in glycosuria in diabetic patients following the administration of alkali. These workers attributed the effects observed to changes in the acid-base equilibrium. Beard ('18) found little or no effect from the giving of sodium bicarbonate, but observed that two of his diabetic patients, who voluntarily took increased amounts of sodium chloride and water, experienced increases in their carbohydrate tolerance. No reference is made in these papers to potassium or to the effect on blood pressure.

More recent studies on the effect of acid-base balance on carbohydrate metabolism are somewhat contradictory. Thompson, Mitchell and Kolb ('33) found in normal subjects that acid administration was followed by diminution of carbohydrate tolerance as indicated by blood sugar curves, while alkalosis had little effect. In a series of short-time experiments on normal children, Johnston and Maroney ('35) more

recently found the oxidation of dextrose to be accelerated on the acid side of neutrality and inhibited on the alkaline side. They also found the oxidation of dextrose to be depressed by the ingestion of sodium chloride and water during the short time of their retention in the body, but found it to be accelerated when the salt and water were released. Of course, these acute experiments on normal subjects cannot be compared directly with our prolonged experiments on diabetic patients. It is unlikely that the neutral NaCl or KCl, as given in our longer experiments, caused any important shift in the acid-base equilibrium.

The somewhat surprising discovery, that potassium exerts effects directly antagonistic to those of sodium, suggests the possibility that the beneficial influence of high sodium intake on carbohydrate metabolism in some way is dependent upon this interrelationship. It has been reported that serum potassium is characteristically elevated in diabetes mellitus. Rathery and Bertoliatti ('34) for instance, found values averaging 35 per cent above normal in seven cases of diabetes not on insulin therapy and 22 per cent above normal in seven insulin-treated cases. Harrop and Benedict ('24) found potassium as well as inorganic phophorus and glucose of blood to be reduced by insulin in normal and diabetic subjects. Briggs and co-workers ('23) found a decrease of 24 per cent in the serum potassium in fasting dogs following the administration of insulin in large doses. Harrop and Benedict suggest that the potassium may enter into the intermediary metabolism of carbohydrate in association with the phosphorus-carbohydrate combination. The few potassium determinations made thus far in our studies indicate that high NaCl ingestion results in a significant decrease in serum K, similar to that occurring after institution of insulin therapy. This phase of the problem is still under investigation. One case is cited above in which severe spontaneous hypoglycemia appears to have been temporarily controlled by administration of KCl in large doses.

The mechanism by which sodium salts result in an increase and potassium salts in a decrease in arterial pressure is unknown. Purely mechanical factors, such as a possible change in plasma volume and change in resistance to blood flow due to accumulation or depletion of fluid in the interstitial fluid reservoir of the body after excessive ingestion of the various salts, may play some role. Measurements of blood volume were attempted in a few instances but the results were inconclusive. Some specific effects either direct or indirect on the arterioles or the kidneys are more likely the important factors. While the observations of McLester ('22), Mosenthal and Short ('23) and others failed to confirm the claims of Allen ('22) that a 'salt free' diet results in a lowering of blood pressure in arterial hypertension, the potassium content of the diets used by the different workers may have differed sufficiently to account for their obtaining conflicting results. We are attempting at the present time to determine the effect of low-sodium, high-potassium diets on young subjects with hypertension.[2]

The possible involvement of certain nervous and endocrine factors are also being investigated by means of animal experiments. Because of the relationships of pituitary and adrenal functions to blood pressure and to the water, mineral and carbohydrate metabolism these glands are receiving first attention.

The cause of marked salt craving in two of our diabetic patients remains obscure. An underlying disturbance in the potassium metabolism was suggested by a violent, anaphylactic-like reaction experienced by L.R. when given potassium chloride in much smaller dosage than that taken without any ill effect whatsoever by other subjects studied.

Studies on the blood changes and on the mineral balances will be presented in subsequent reports.

[2] After the present paper was set up for printing, the authors discovered a paper by W. L. T. Addison (Can. Med. Assoc. J., 1928, vol. 18, p. 281) describing effects of Na and K salts on the blood pressure of adult patients with arterial hypertension, which were similar to those recorded here for non-hypertensive children.

SUMMARY

1. The effects of ingesting excessive quantities of sodium and potassium salts on the blood pressure and carbohydrate metabolism have been studied in one non-diabetic and four diabetic children. Repeated tests were made in the case of each diabetic subject. While the most striking effects were observed in the case of one particular diabetic, who consistently required between 60 and 90 gm. of NaCl daily to satisfy his craving for salt, all patients submitted to the tests responded in like manner.

2. The daily ingestion of between 1 and 2 gm. of NaCl per kilogram of body weight resulted within a period of from 2 to 4 days in a gain of from 4 to 5 per cent in body weight and an increase in both systolic and diastolic blood pressure to new plateaus between 30 and 50 per cent above the control levels. These higher pressures were maintained so long as the salt was taken.

3. In addition to this effect on blood pressure, the excessive ingestion of NaCl was found to cause a marked reduction in the degree of glycosuria in the diabetic subjects studied. The effects were qualitatively the same in one mildly diabetic patient not given insulin as in those patients who received insulin at regular 6-hour intervals.

4. The fasting blood sugar was found to range at lower levels after a few days of the high NaCl ingestion than during the foreperiod, the patient's food intake, insulin dosage and activity remaining unchanged. The fasting R.Q. was slightly higher during the period of high NaCl intake than previously. The shape of the sugar tolerance curve, however, was not measurably influenced by salt ingestion.

5. In severe diabetes, ketonuria appeared earlier after withdrawal of insulin when the NaCl intake was low than when it was very high.

6. With a constant protein intake estimated to maintain nitrogen balance under ordinary conditions the nitrogen output exceeded the intake during periods of low NaCl intake. During periods of high NaCl intake, however, the nitrogen balance became positive.

7. Typical insulin reactions were found to occur at times during the period of high NaCl intake in patients receiving insulin in dosages found during the low-salt periods to be sufficient to completely prevent glycosuria.

8. Sodium bicarbonate and sodium citrate when given in amounts with equivalent sodium values had similar though less marked effects than those of NaCl.

9. The maximum effects of NaCl on both the B.P. and the carbohydrate metabolism were obtained only when the patients were given simplified diets which were low in potassium. Ordinary diets, high in potassium, either prevented or greatly lessened the effects of the NaCl.

10. Potassium chloride, when given in doses of 10 to 20 gm. daily along with a simplified diet low in sodium, resulted in a slight fall in both systolic and diastolic blood pressure and at the same time a significant increase in the degree of glycosuria, thus exerting effects diametrically opposite those of the sodium salt.

11. When the two salts were given simultaneously one part of potassium was found to completely abolish the effects of at least three chemically equivalent parts of sodium.

12. A small number of serum potassium determinations have been made. The high NaCl intake depresses the serum potassium.

CONCLUSIONS

1. When ingested in amounts varying between 1 and 2 gm. per kilogram of body weight daily, sodium chloride exerts a favorable influence on the carbohydrate metabolism of diabetic children taking simplified diets low in potassium. This effect is observable usually on the second or third day.

2. At the same time both the systolic and diastolic blood pressure levels are elevated significantly.

3. Sodium appears to be chiefly responsible for these effects since other salts of this element as well as the chloride exert similar, though less marked, effects.

4. Potassium chloride has diametrically opposite effects on both glycosuria and blood pressure.

5. In terms of chemical equivalents, potassium completely antagonizes the effects of sodium when given simultaneously in amounts as little as one-third that of sodium.

6. The physiological mechanisms involved in these reactions are at present obscure but are being further investigated.

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THE RELATIVE VITAMIN A POTENCY OF CAROTENE FED IN BUTTER FAT AND COTTONSEED OIL [1, 2]

HENRY R. KRAYBILL AND CHARLES L. SHREWSBURY
Departments of Agricultural Chemistry and Animal Husbandry, Purdue University Agricultural Experiment Station, Lafayette, Indiana

TWO CHARTS

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The amounts of vitamin activity due to the carotene and vitamin A respectively of butter fat cannot be determined with precision at present. In the work that has been reported on this subject (Shrewsbury and Kraybill, '33; Baumann and Steenbock, '33; Baumann, Steenbock, Beeson and Rupel, '34; Booth, Kon, Dann and Moore, '33; and Gillam, '34) certain assumptions have been made with respect to the relative potency of equal quantities of carotene and vitamin A. Even though we know the amounts of carotene and vitamin A in butter fat, in order to determine the vitamin potency of each of these constituents, it is necessary to know their relative vitamin potency when fed in butter fat.

Dutcher, Harris, Hartzler and Guerrant ('34) found that the presence of mineral oil lowered the vitamin potency of carotene but did not affect adversely the vitamin potency of cod liver oil and a cod liver oil concentrate. The adverse effect of mineral oil in the case of carotene was explained on the basis of carotene excretion from the body in the unabsorbed oil. Lathbury and Greenwood ('34) report that different batches of arachis oil and coconut oil vary in their

[1] Wesson oil, a refined cottonseed oil.

[2] Presented at the meetings of the American Chemical Society, April, 1935, New York City.

suitability as carriers for the biological testing of vitamin A or carotene and that the suitability of any oil is not entirely dependent on the stability of the dissolved substances in the oil. They make the interesting observation that some samples of arachis oil are better than coconut oil while others are not so good as solvents for testing carotene or vitamin A. They point out further that it is not unlikely that certain oils contain a factor necessary to supplement highly purified concentrates of vitamin A and carotene but that this factor is apparently not present in oils of any one kind.

Dyer, Key and Coward ('34) found that a solution of international standard carotene in arachis oil had from five to six times the potency of similar solutions in hydrogenated cottonseed oil and that one sample of cod liver oil dissolved in coconut oil had three times the potency as when dissolved in hydrogenated cottonseed oil.

Utilization of carotene[3] *from charcoal decolorized butter fat*

In a previous paper (Shrewsbury and Kraybill, '33) from this laboratory we have shown that butter fat treated with charcoal loses its color, vitamin A activity and antioxidants. Carotene added to this butter fat containing hydroquinone is quite stable.

Experiments in which carotene was fed in charcoal decolorized butter fat indicated that at least 2 micrograms per day were required for one Sherman unit (3 gm. average gain per week for 8 weeks). This amount is four times the amount of carotene administered in cottonseed oil found to be necessary for one Sherman unit. This was true even when the dose of carotene and butter fat was prepared fresh daily, or when hydroquinone was added to the preparation to prevent oxidation of carotene.

[3] The carotene used in these studies was obtained from the S.M.A. Corporation, Cleveland, Ohio. We are indebted to Doctors Miller, Zscheile and Hogness of the chemistry department of The University of Chicago for the following analysis of the carotene: Beta carotene, 81.0 per cent; alpha carotene, 13.0 per cent; colorless impurities, 6.0 per cent.

Since the color of charcoal decolorized butter fat containing added carotene and hydroquinone was found to be stable by spectrophotometric measurements the question arose as to whether hydroquinone affected the utilization of carotene in the animal body.

Several samples of cottonseed oil containing from 2.0 to 100 mg. of hydroquinone per 100 cc. of oil were prepared. Carotene was added to all of these in an amount that would furnish 1 microgram in 5 drops of cottonseed oil. These materials were fed to rats depleted of their vitamin A stores.

TABLE 1

The effect of hydroquinone on the utilization of carotene in cottonseed oil

MILLIGRAMS HYDROQUINONE PER 100 GM. COTTONSEED OIL	CAROTENE IN DAILY DOSE MICROGRAMS	NUMBER OF ANIMALS STARTED ON EXPERIMENT	NUMBER OF SURVIVORS	SEX DISTRIBUTION	AVERAGE WEEKLY GAIN IN GRAMS AT END OF			
					Fourth week	Fifth week	Sixth week	Eighth week
				♂ ♀				
None	1.0	5	5	3—2	7.5	7.4	6.9	6.3
2.0	1.0	5	5	4—1	7.2	7.6	7.3	6.9
10.0	1.0	4	3	1—3	9.2	8.0	8.1	9.5
25.0	1.0	4	4	1—3	8.5	8.8	8.2	7.7
50.0	1.0	4	4	1—3	8.2	8.4	8.1	7.3
100.0	1.0	3	3	2—1	5.7	5.1	5.0	5.8

The experimental technic used in these experiments was the same as previously described (Shrewsbury and Kraybill, '33). The results are given in table 1.

There is no significant interference with the utilization of carotene by hydroquinone.

Decolorization of butter fat with Lloyd's reagent

A number of absorbents were examined with the hope of finding one that would remove the vitamin A activity and leave the antioxidants undisturbed. Lloyd's reagent proved to be quite efficient in this respect.

An absorption tube 6 inches long by 1 inch in diameter was filled to about three-fourths capacity with Lloyd's reagent. A plug of cotton was placed in the bottom of the tube

and the Lloyd's reagent packed in firmly by introducing small quantities at a time and tapping the tube. This tube was attached to a side neck suction flask. The butter was melted and drawn through the column with the aid of suction. The rate of flow through the Lloyd's reagent was slow and it was necessary to keep the material warm (below 50°C.) to prevent solidification of the butter. The butter fat was passed through the column twice in most cases. This treatment removed practically all of the color and the vitamin A content of the butter fat (see chart for vitamin A activity). However,

TABLE 2

Stability of carotene in butter fat decolorized with Lloyd's reagent. Materials stored at 40°C. Expressed as milligrams of carotene per 100 gm. of fat

SAMPLE	FRESH SAMPLE	SAMPLE AFTER STORAGE	STORAGE TIME IN DAYS
1	0.65	0.63	14
2	0.55	0.63	20
3	0.53	0.55	16
4	0.67	0.55	16
5	1.13	1.00	12
6	1.01	0.92	56
7[1]	0.47	0.47±0.005	60
8	0.49	0.54	10

[1] Sample number 7 was prepared by adding carotene equivalent to 0.5 mg. per 100 gm. of butter fat. We are indebted to Doctors Miller, Zscheile and Hogness of the chemistry department of The University of Chicago for making this analysis by spectro-photoelectric methods. (Plant Physiology, '34, vol. 9, p. 681.)

unlike butter fat treated with charcoal that decolorized with Lloyd's reagent retained antioxidant properties. The stability of carotene added to butter fat decolorized with Lloyd's reagent is demonstrated by the results of spectrophotometric readings given in table 2.

Because of the slow rate of flow of butter fat through the Lloyd's reagent column another method of decolorization was tried. Fifty grams of Lloyd's reagent was allowed to stand in contact with 200 gm. of melted butter fat in a stoppered Erlenmeyer flask at 50°C. for about 8 hours. The mixture was agitated from time to time. The Lloyd's reagent was

filtered off through a Buchner funnel fitted with a qualitative filter paper. The funnel was kept warm with a hot water coil. For complete decolorization it was usually necessary to repeat the treatment with Lloyd's reagent. Butter fat prepared in this manner contained no appreciable color or viamin A potency but retained antioxidant properties.

Extraction of the Lloyd's reagent used to decolorize butter fat, with ether followed by alcohol yields a part of the color. In one experiment 11.63 per cent of the original coloring matter of butter was recovered by ether extraction and 18.15 per cent by extraction with 95 per cent alcohol. A total of 39.78 per cent of the color was recovered.

Extraction of the charcoal used to decolorize butter with ether and alcohol did not effect recovery of any of the color. It seems that in the case of charcoal the reaction completely destroys or removes the carotene and antioxidants presumably by oxidation while apparently only slight losses occur when Lloyd's reagent is used. This may explain the antioxidant properties of butter prepared by treatment with Lloyd's reagent and the lack of antioxidant properties in charcoal decolorized butter.

Utilization of carotene from Lloyd's reagent decolorized butter fat

Since carotene added to butter fat decolorized with Lloyd's reagent is quite stable this material was used to study the utilization of carotene from butter fat. Varying amounts of carotene (from 0.5 to 4.0 micrograms) were fed to rats in from 50 to 400 mg. of decolorized butter fat and also in similar quantities of cottonseed oil. The feeding samples were prepared fresh every 2 weeks throughout the feeding period of 8 weeks. The data are given in charts 1 and 2.

These data demonstrate that there is a considerable difference in the vitamin potency of carotene when fed in cottonseed oil and when fed in butter fat. A daily dose of 0.5 microgram of carotene in cottonseed oil per rat produced approximately 3 gm. gain per week for 8 weeks. The mortality was low; eleven of the thirteen rats starting in the

experiment survived. An increase in the carotene dosage caused an increase in gain roughly proportional to the amounts administered. The cottonseed oil contained negligible quantities of vitamin A. Twenty drops of plain cottonseed oil per rat daily furnished no better protection against vitamin A deficiency than 5 drops. The survival period,

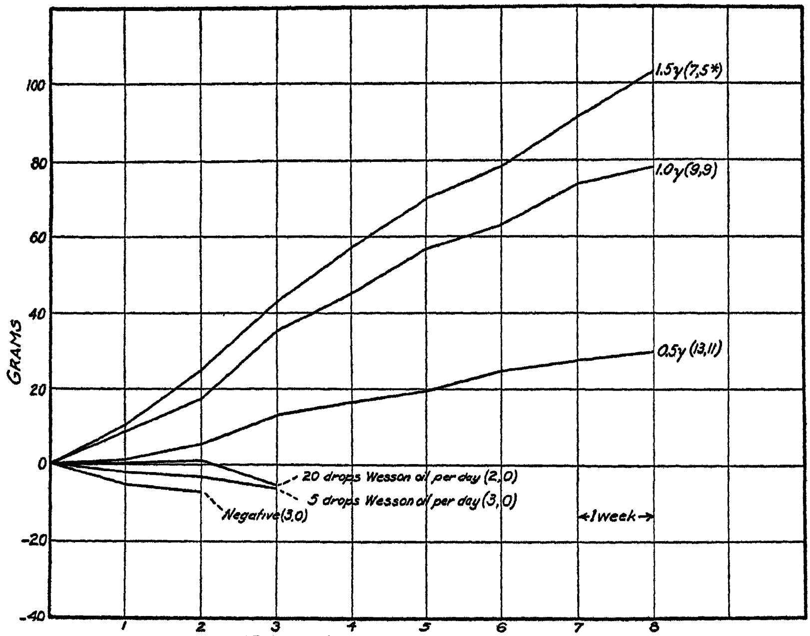

CHART 1. CAROTENE FED IN WESSON OIL

Chart 1 Figures on curves indicate in micrograms the daily dose of carotene per rat. Figures in parentheses indicate the number of animals started in the experiment and the survivors. * Two animals removed to conserve carotene.

however, was slightly longer than obtained with the negative controls.

One-half microgram of carotene per rat per day fed in decolorized butter fat produced practically no gain in weight and the mortality was high. Only one animal of fourteen finished the experiment alive. When the dosage was increased to 1.0 microgram per rat per day the growth was nearly equal to that produced by 0.5 microgram of carotene fed in cottonseed oil but the mortality was high. Not until 2.0 micrograms

of carotene per rat per day were fed was the mortality reduced to a point comparable to the mortality on 0.5 microgram of carotene fed in cottonseed oil. Growth at this level was more rapid. Decolorized butter fat had little or no vitamin A activity. As much as 1.0 gm. of this butter fat per rat per day maintained vitamin A depleted rats only slightly longer than the unsupplemented basal diet.

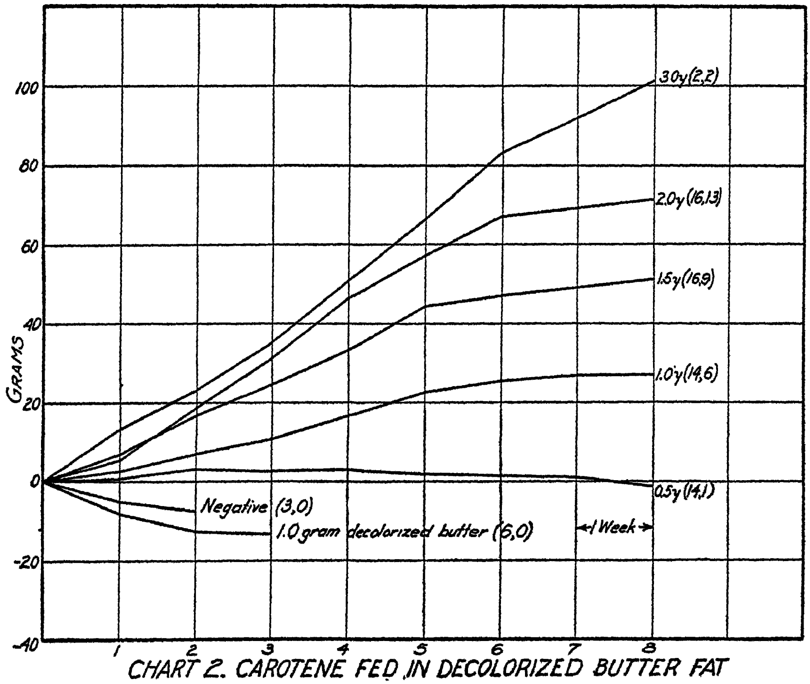

Chart 2 Figures on curves indicate in micrograms the daily dose of carotene per rat. Figures in parentheses indicate the number of animals in the experiment and the survivors.

The high mortality of the animals that received carotene dissolved in decolorized butter fat even at relatively high levels of carotene intake, and which were making rapid gains suggests that the decolorization with Lloyd's reagent may have removed a factor which supplemented the vitamin A activity of carotene.

If this assumption were true it might account, in part at least, for the difference between the utilization of carotene from cottonseed oil and from the decolorized butter fat. This problem is now under investigation.

CONCLUSIONS

1. Treatment of melted butter fat with Lloyd's reagent removes the natural yellow pigments and vitamin A without noticeable destruction or removal of the natural antioxidants.
2. Hydroquinone in amounts as high as 100 mg. per 100 gm. of oil does not seriously interfere with the utilization of carotene from cottonseed oil.
3. Carotene dissolved in butter fat decolorized with Lloyd's reagent is not utilized as well as when it is dissolved in cottonseed oil. Two to three times as much carotene are required to give equal vitamin A potency when fed in butter fat decolorized with Lloyd's reagent as when fed in cottonseed oil.

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INFLUENCE OF SOIL AND VARIETY ON THE COPPER CONTENT OF GRAINS [1]

J. E. GREAVES AND ARIEL ANDERSEN
Agricultural Experiment Station, Logan, Utah

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That copper occurs in plants has been known for over a century (Meissner, 1816), but for a long time it was generally considered to be an accidental contaminant. Maquenne and Demoussy ('20) found from 3 to 40 parts per million in all plant material they analyzed and suggested that copper must be an essential element in plant metabolism. Since then numerous contributions have substantiated this view and demonstrated that copper also plays a vital role in animal metabolism (Elvehjem, '35). These discoverers have given to analytical studies of copper content of plants new importance. It has been shown that the ash content of Utah-grown grains is not only high but varies depending upon locality, variety, and cultural conditions (Greaves and Hirst, '29 a). The present study was undertaken to determine, if possible, if the same factors control the copper content of grains.

METHODS

Samples of grains and corresponding soil samples were collected from various localities throughout the state of Utah. These were prepared for analyses according to the methods of The Association of Official Agricultural Chemists ('30). The grains were analyzed for copper according to the modified Biazzo method of Elvehjem and Lindow ('29). After

NOTE: The varieties of wheat analyzed were furnished by Prof. A. F. Bracken and were grown on the Nephi Dry-farm Substation.

[1] Contribution from department of chemistry and bacteriology, Utah Agricultural Experiment Station. Publication authorized by director, October 7, 1935.

considerable experimentation and careful checking which guaranteed reliable results, the following method was adopted for determining the copper content of the soil.

Thirty grams of soil were weighed into 500 cc. Erlenmeyer flasks, 60 cc. concentrated nitric acid and 5 cc. of concentrated hydrochloric acid being added and allowed to stand over night. It was then boiled for 30 minutes, diluted with water, filtered through a procelain filter, and washed to 400 cc. The filtrate was evaporated to dryness, from 3 to 5 cc. of concentrated hydrochloric acid added, and again evaporated to dryness. This process was repeated until practically all nitric acid was removed. Twenty cubic centimeters hydrochloric acid (1:1) were added, the solution warmed and washed into a 250 cc. flask with 125 cc. of hot water. The solution was then heated to boiling and hydrogen sulfide passed through until cold. The flask was stoppered, allowed to stand 1 hour, and tested for hydrogen sulfide saturation. The solution was filtered through paper pulp over a fine grade of filter paper and washed free of iron with approximately 100 cc. 0.3 normal hydrochloric acid saturated with hydrogen sulfide. The precipitate and filter paper were ashed and the residue taken up with 3 drops of nitric acid (1:9), 2 drops of hydrochloric acid (1:9), and 10 drops of concentrated sulfuric acid; this was heated to sulfuric acid fumes, the residue being made up to 25 cc. with distilled water and the copper determined in an aliquot by the modified Biazzo method of Elvehjem and Lindow ('29). The method, as outlined, was first checked on soils of known copper content and was found to recover over 98 per cent of the added copper. The water, chemicals, and apparatus were all checked for copper. All determinations were made in duplicate or triplicate and averages only are given.

RESULTS

Table 1 gives the parts per million of copper found in wheat and soil from various sections of the state.

Copper content of Utah wheats analyzed varied from 5.8 to 10.2 parts per million, with an average of 7.8. Webster

and Jansma ('29) reported the copper content of wheat from a number of states as varying from 4.2 to 8.7 parts per million, with an average of 6.0. This places Utah wheat slightly higher in copper content than for other states reported. Invariably the copper content of the soil was found to be higher than that of the wheat grown upon it. The copper content of the soil varied from 6.4 to 24.5, with an average of 16.7 parts per million. Hence, there was no concentration of the copper in the seed, as is usually the case with essential elements.

Deschamps, as early as 1848, suggested that a definite relationship existed between the copper content of plants and the copper content of the soil in which they were grown.

TABLE 1

Parts per million copper found in soil and wheat from different parts of Utah

SAMPLE LOCATION	COPPER CONTENT (P.P.M.)		SAMPLE LOCATION	COPPER CONTENT (P.P.M.)	
	Soil	Wheat		Soil	Wheat
Farmington	24.5	8.1	Howell	16.7	8.1
Fairview	22.7	8.7	Joseph	15.8	9.7
Fairview	22.7	10.2	Redmond	13.9	7.9
Cedar Valley	19.6	5.8	Hansel Valley	13.3	10.2
Utah County	18.9	6.3	Richfield	8.3	7.2
Utah County	17.5	6.3	Santa Clara	6.4	5.9
Cedar Valley	17.2	6.9			

Flinn and Inouye ('29), however, consider the copper content of plants to be due to a physiological factor and not related to the copper content of the soil.

Calculations, in regard to the coefficient of correlation between soil and wheat, were made according to the formula $r = \frac{\Sigma x y}{\sqrt{\Sigma x^2 \Sigma y^2}}$ which was 0.273, with a probable error of correlation 0.363. This indicates no relationship between copper content of the wheat and soil. Average results for copper determinations made on barley are given in table 2.

Samples of wheat, oats and barley were often taken from different localities, which accounts for the variation in the copper content of soil from the same farming district. The copper content of Utah barley analyzed varied from 6.2 to

11.9 parts per million, with an average of 7.8. Again, the soil was invariably higher in copper than was the grain and varied from 6.4 to 41.2 parts per million of copper with an average of 17.6. The coefficient of correlation of 0.136, with a probable error of 0.204, indicates no correlation between the copper content of soil and of grain.

Average results of copper determinations made on samples of oats are given in table 3.

TABLE 2

Parts per million copper found in soil and barley, Utah

SAMPLE LOCATION	COPPER CONTENT (P.P.M.)		SAMPLE LOCATION	COPPER CONTENT (P.P.M.)	
	Soil	Barley		Soil	Barley
Farmington	41.2	8.4	Joseph	13.5	9.8
Eden	27.4	6.5	Upper Hansel Valley	13.2	6.7
Fairview	22.7	6.5	Utah County	12.6	7.0
St. George	17.5	11.9	St. George	7.6	6.8
Redmond	14.3	8.0	Richfield	6.4	6.2

TABLE 3

Parts per million of copper found in soil and oats, Utah

SAMPLE LOCATION	COPPER CONTENT (P.P.M.)		SAMPLE LOCATION	COPPER CONTENT (P.P.M.)	
	Soil	Oats		Soil	Oats
Farmington	50.9	6.4	Utah County	13.1	8.5
Joseph	19.2	7.5	St. George	12.5	7.4
Eden	18.5	6.5	Santa Clara	4.1	6.8
Redmond	14.1	6.5	Richfield	3.9	9.8

The copper content of Utah oats analyzed varied from 6.4 to 9.8 parts per million, with an average of 7.4. Therefore, insofar as this survey goes, it tentatively may be concluded that the average copper content of wheat, oats and barley is practically the same. The soils on which oats were grown carried from 3.9 to 50.9 parts per million copper, with an average of 17.0. The coefficient of correlation is —0.478, with a probable error of 0.182. It was only in the case of the soils containing less than 6.4 parts per million of copper that a concentration of copper is found in the grain. Does

the copper requirement of grain approximate this figure? Sommer ('31), Lipman and Mackinney ('31) grew plants on synthetic media and found that they would neither mature nor produce seeds without copper. This they considered essential in all phases of plant growth; they found from $\frac{1}{16}$ to $\frac{1}{8}$ part per million sufficient for growth and production of seeds but considered that more was necessary for normal crop production.

Webster and Jansma ('29) compared the copper content of wheat from several states and found it to range from 4.2 to 8.7 parts per million of copper. According to these investigators there seems to be no relation between ash content and copper content of grain. Flinn and Inouye ('29) contend that the copper content of the plant bears no relation to the copper content of the soil. This is probably true where the copper content of the soil exceeds 6 parts per million. However, they postulate a relationship between the copper content of the plant and certain physiological properties. The protein and ash content of grains varies widely with the variety (Greaves and Hirst, '29 b). Will this also hold in the case of copper? In order to answer this question, sixteen varieties of wheat, grown on the same soil and under similar conditions, were analyzed for copper. Each yearly sample was composed of three replications; the samples used for analyses were further composited from wheat grown during each of 6 years. Each analyzed sample was a composite of eighteen samples. Results, as given in table 4, represent the averages of three or more closely agreeing determinations from such a composite sample.

There is a wide variation in the copper content of different varieties of wheat. Montana 36, although grown on the same soil and under similar conditions, had three times the copper content of Kofod x Turkey.

Wheat collected from various parts in the state varied from 5.8 to 10.2 parts per million of copper, with an average of 7.8. The sixteen varieties of wheat all grown under similar conditions showed variation in copper content from 5.6 to 16.7

parts per million, with an average of 9.7. Therefore, it would seem that variety is more important than soil, insofar as copper content of grains is concerned. However, where the copper content of the soil falls below a certain minimum, this may not necessarily hold. There are a number of cases on record where the copper content of plants has been increased by the use of copper fertilizers (Elvehjem and Hart, '29; Miller and Mitchell, '31). Lindow, Elvehjem and Peterson ('29) analyzed 158 foods and found none completely lacking in copper. Their figures range from 0.1 mg. of copper per kilogram of fresh celery to 44.1 mg. per kilogram of fresh calf liver. Foods were classified in descending order of copper content, as follows: Nuts, dried legumes, cereals, dried fruits, poultry, fish, animal tissue, green legumes, roots and tubers, leafly vegetables, fresh fruits, and non-leafly vegetables.

TABLE 4

Parts per million of copper found in different varieties of wheat, Utah, grown on same soil

VARIETY OF WHEAT	COPPER CONTENT (P.P.M.)	VARIETY OF WHEAT	COPPER CONTENT (P.P.M.)
Montana 36	16.7	Karamont	7.8
Kubanka	16.4	Black Hull	7.6
Turkey 926	13.9	Hard Federation	7.4
Tenmarq	12.3	Marquis	7.0
Kharkov Hayes 2	11.8	Kofod	6.5
Newturk	11.0	Alton	6.4
Regal	9.4	Chul	5.9
Early Baart	9.3	Kofod x Turkey	5.6

Although there are districts in which the copper content of the soil is extremely low (Russell and Manns, '33) and the quantity occurring in the plants low enough to cause deficiency diseases in animals feeding solely upon them (McHargue, '25; Neal and Becker, '33; Sjollema, '33), yet the limited data presented in this paper point at least to the tentative conclusion that this is not to be expected in Utah.

SUMMARY

Wheat, oats and barley, together with the corresponding soils from different parts of the state, were analyzed for copper. The wheat varied from 5.6 to 16.7 parts per million copper, with an average of 8.8. The barley varied from 6.2 to 11.9 parts per million, with an average of 7.8. The oats varied from 6.4 to 9.8 parts per million, with an average of 7.4. Corresponding soils carried from 3.9 to 50.9 parts per million, with an average of 17.1 parts per million. The copper content of the grain was invariably lower than that of the soil until the copper content of the soil was below 6 parts per million. No correlation was found between the copper content of the grain and the soil on which it was grown.

Sixteen varieties of wheat grown on the same soil and under similar conditions varied in copper content from 5.6 to 16.7 parts per million, with an average of 9.7. It therefore appears probable that variety is the main factor in determining the copper content of Utah-grown wheats. It appears improbable from the limited data presented in this paper that copper is a limiting factor in plant or animal nutrition, insofar as Utah is concerned.

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THE VITAMIN A RESERVE OF EMBRYO AND BABY CHICKS

ARTHUR D. HOLMES AND FRANCIS TRIPP
The E. L. Patch Company, Research Laboratories, Boston

AND

PERCY A. CAMPBELL
Eastern States Farmers' Exchange, Research Department, Springfield, Mass.

TWO CHARTS

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It has been noted by Emmett and Peacock ('23), Beach ('24), Hart, Steenbock, Lepkovsky and Halpin ('24), Hauge, Carrick and Prange ('27), Holmes, Pigott and Menard ('30), Heywang and Titus ('32), Guilbert and Hinshaw ('34), Frohring and Wyeno ('34) and others that chick rations deficient in vitamin A produce unsatisfactory growth and in order to obtain optimum growth a chick ration should be rich in vitamin A. Hence those responsible for compounding chick growing rations ordinarily attempt to insure an adequate supply of vitamin A, by including in the ration a number of vitamin A rich products, with the hope that sufficient vitamin A will be present to meet all probable needs for growth. Obviously this procedure, which relies upon maintaining a large excess of vitamin A in chick growing rations, entails unnecessary waste. Unfortunately those who compound chick rations do not have definite information concerning the vitamin A requirements of growing chicks. Furthermore a review of the literature revealed little information concerning either the size of a baby chick's store of vitamin A or the amount of vitamin A that a normal chick requires for optimum growth. Accordingly a study has been

made of the vitamin A reserve of chicks just before, at, and subsequent to hatching.

For the purpose of this study 100 embryo and 298 young chicks of two breeds, Rhode Island Red (R.I.R.) and Barred Plymouth Rock (B.P.R.) were obtained from six sources. In determining the vitamin A reserve of embryo and young chicks attention was centered on the livers for Baumann, Riising and Steenbock ('34) have reported that 95 per cent of the total vitamin A store of rats is present in the liver and Sherman and Boynton ('25), Rosenheim and Webster ('27), Moore ('31), McCoord and Luce-Clausen ('34) and others have obtained similar results. Since the size of chick livers at the end of incubation did not permit of individual analyses the livers from ten chicks were pooled. The vitamin A content of the livers was determined by the Carr-Price ('26) antimony trichloride colorimetric method. As soon as the livers were removed they were ground, as suggested by Torrance ('33), with an equal weight of anhydrous sodium sulphate. The mixture was extracted 8 hours with anhydrous ether. The ether was volatilized and the resulting fat covered with an atmosphere of nitrogen. A weighed amount of the fat was dissolved in chloroform and the vitamin assay was made by the usual procedure.

The first determination of the vitamin A store of embryo chicks (R.I.R. and B.P.R.) was made with dead embryos (hatchery 'W') obtained at the end of the incubation period. Since it is a routine practice to remove all dead embryos from the incubator on the eighteenth day of incubation the embryos in question were 19 to 21 days old. Inasmuch as the unabsorbed yolk contains a store of vitamin A it was decided to determine the vitamin A content of both livers and unabsorbed yolks. Unfortunately it was impossible to dissect the unabsorbed yolks from the first three groups of embryos. The fat content of the livers from the six groups of dead embryos (table 1) varied from 11.5 per cent to 16.0 per cent. The average vitamin A content for the livers of the six groups varied from 8 to 14 blue units. The average fat content of

the unabsorbed yolks was 18.6 per cent. The blue units for three groups of yolks varied from 22 to 35 units per yolk.

Since exact information was not available concerning the time of death of the embryos it was decided to determine the vitamin A reserve of live embryos of known age. Accordingly embryos of two breeds (R.I.R. and B.P.R.) were obtained (hatchery 'S') when the embryos were 18 days old. The parent stock which produced the embryo chicks all received

TABLE 1

Vitamin A reserve of embryo chicks[1]

BREED AND SOURCE	AGE	WEIGHT OF CHICK	WEIGHT OF LIVER	LIVER FAT	BLUE UNITS PER LIVER	WEIGHT OF YOLK	YOLK FAT	BLUE UNITS PER YOLK	TOTAL BLUE UNITS OF LIVER AND YOLK
Dead embryo									
	days	*gm.*	*gm.*	*per cent*		*gm.*	*per cent*		
RIR-W	19–21	..	0.92	14.3	14	....	....	..	..
RIR-W	19–21	..	0.91	13.6	13	....	....	..	..
RIR-W	19–21	41	0.87	14.6	8	....	....	..	..
RIR-W	19–21	40	1.02	16.0	13	7.4	17.7	22	35
BPR-W	19–21	41	0.95	15.0	14	7.6	21.4	32	46
BPR-W	19–21	41	0.87	11.5	8	11.5	16.6	35	43
Average (3)		41	0.92	14.2	12	8.8	18.6	30	41
Live embryo									
RIR-S	18	..	0.47	10.7	9	15.0	27.0	87	96
RIR-S	18	..	0.55	13.8	6	13.5	26.4	76	82
BPR-S	18	..	0.47	10.9	8	14.9	27.1	79	87
BPR-S	18	..	0.58	14.7	6	13.6	26.2	76	82
Average		..	0.51	12.5	7	14.3	26.7	80	87

[1] Average values—groups of ten chicks.

the same ration and was maintained under the same environment. The livers from these embryos were about one-half as large as those of the older embryos and contained about 1½ per cent less of fat. The weight of the unabsorbed egg yolks, 14.3 gm., indicates that approximately one-fifth of the yolk had been absorbed by the eighteenth day of incubation. The results of the vitamin A assays of the livers and unabsorbed yolks of the four groups of 18-day embryo chicks show the livers to have an average value of 7 blue units and the unabsorbed yolks 80 blue units.

Attention was next given to the vitamin A store of chicks at the time of hatching. Three groups of baby chicks were removed from the incubator as soon as possible after hatching and killed when approximately 6 hours old. The average weight of the livers (table 2) from the thirty chicks was 0.89 gm. and they contained 17.2 per cent of fat. The average weight of the unabsorbed yolk was 8.3 gm. In other words about 55 per cent of the original yolk had been absorbed, indicating that 50 per cent more yolk was absorbed during the last 3 days than during the first 18 days of incubation.

TABLE 2

Vitamin A reserve of baby chicks[1]

BREED AND SOURCE	AGE	WEIGHT OF CHICK	WEIGHT OF LIVER	LIVER FAT	BLUE UNITS PER LIVER	WEIGHT OF YOLK	YOLK FAT	BLUE UNITS PER YOLK	TOTAL BLUE UNITS OF LIVER AND YOLK
	days	*gm.*	*gm.*	*per cent*		*gm.*	*per cent*		
RIR-S	0.25	44	0.88	17.9	10	8.8	25.7	57	67
RIR-W	0.25	44	0.94	16.4	17	8.1	22.2	68	85
BPR-S	0.25	43	0.86	17.2	8	7.9	24.6	50	58
Average		44	0.89	17.2	12	8.3	24.2	58	70
RIR-H	1	40	1.16	20.3	27	4.0	15.0	34	61
RIR-S	1	40	1.09	18.3	10	6.1	23.4	47	57
RIR-W	1	35	1.16	17.2	14	4.9	17.3	39	53
BPR-S	1	41	0.98	18.8	13	6.7	25.1	62	75
BPR-W	1	36	1.09	19.2	29	4.0	11.3	39	68
Average		38	1.10	18.8	19	5.1	18.4	44	63

[1] Average values—groups of ten chicks.

The vitamin A content of the livers and unabsorbed yolks of the 6-hour-old chicks from hatchery 'W' was materially larger than that of livers and yolks from hatchery 'S' chicks. According to the work of Sherwood and Fraps ('32) it is possible that this larger store of vitamin A may result from a difference in the vitamin A content of the rations of the breeding stock that produced the eggs for the two hatcheries. Bethke, Kennard and Sassaman ('27) and Ellis, Miller, Titus and Byerly ('33) found that the addition of cod liver oil to the ration of laying hens materially increased the vitamin A content of the eggs produced. It is also possible that the

vitamin A content of the eggs used by the two hatcheries may have been influenced by the rate of production of the breeding stock. Koenig, Kramer and Payne ('35) found 33 units of vitamin A per gram of yolk for low-producing birds as compared with 20 units for high-producing birds. The average vitamin A store of the 6-hour-old chicks was 12 blue units for the liver and 58 blue units for the unabsorbed yolk. Thus the combined liver and unabsorbed yolk store of these chicks was 70 blue units as compared with 80 blue units for 18-day-old embryos.

Five groups of chicks of two breeds obtained from three different hatcheries were killed 24 hours after hatching. The average weight of livers was 1.10 gm. The fat content of the livers was 18.8 per cent which is approximately 1.50 per cent higher than that of comparable 6-hour-old chicks. The weight of unabsorbed yolk, however, was found to be 5.1 gm. or approximately only two-thirds the weight of unabsorbed yolk of the chicks which were killed when 6 hours old. The fat content of the unabsorbed yolk had also decreased during the first day of life from 24.2 per cent to 18.4 per cent. The vitamin A assay of the livers and unabsorbed egg yolk of the 24-hour-old chicks gave an average value of 19 blue units for the livers and 44 blue units for the unabsorbed yolks. These data show a continued decrease of the baby chick's vitmain A reserve.

In order to determine the influence of the absorption of the egg yolk and the early utilization of food by baby chicks on their reserve of vitamin A, two lots of forty Rhode Island Red chicks were obtained from two different sources. The chicks from hatchery 'W' were obtained when approximately 6 hours old and divided into four groups of ten chicks each. One group was immediately killed and the remaining groups were killed, 1, 2 and 3 days later. The first three groups were not fed. The fourth group received mash and water at the end of the second day. The forty chicks from hatchery 'R' were not obtained until they were 2 days old. One group was killed at that time and the remaining groups were killed

1, 2 and 3 days later. The first two groups of chicks were not fed. After the third day mash and water was continuously available to the last two groups of chicks.

It will be noted (table 3) that the body weight of the chicks decreased until the chicks were fed. Thereafter their body weight increased. On the other hand the weight of the liver increased progressively with the age of the chicks. The weight of the unabsorbed yolk decreased from approximately 8 gm. at hatching to 0.6 gm. when the chicks were 5 days old. The vitamin A assay of the livers and the unabsorbed yolk showed a progressive increase in the vitamin A content of the liver

TABLE 3

Decrease in vitamin A following hatching [1]

BREED AND SOURCE	AGE	WEIGHT OF CHICK	WEIGHT OF LIVER	LIVER FAT	BLUE UNITS PER LIVER	WEIGHT OF YOLK	YOLK FAT	BLUE UNITS PER YOLK	TOTAL BLUE UNITS OF LIVER AND YOLK
	days	*gm.*	*gm.*	*per cent*		*gm.*	*per cent*		
RIR-W	0.25	44	0.94	16.4	17	8.1	22.2	68	85
RIR-W	1	40	1.12	19.8	17	5.0	22.8	50	67
RIR-W	2	38	1.18	19.8	19	3.8	15.5	33	52
RIR-W[2]	3	43	1.54	16.2	34	2.1	14.0	45	79
RIR-R	2	36	1.12	18.5	35	2.9	13.6	45	80
RIR-R	3	33	1.13	19.4	42	1.6	10.0	30	72
RIR-R[3]	4	44	1.57	14.1	36	0.9	13.0	40	76
RIR-R	5	48	1.82	11.4	59	0.6	13.9	22	81

[1] Average values—groups of ten chicks.
[2] Received feed at end of second day.
[3] Received feed at end of third day.

and a decrease in the vitamin A contained in the unabsorbed yolk.

In order to estimate the value of the unabsorbed yolk as a reserve source of vitamin A for utilization by the chick during the first 5 days of life, the data obtained from the assay of the unabsorbed yolk from the 198 chicks between the first and fifth day of life have been correlated. The average values obtained from these correlated data are reported as graphs (chart 1). Inspection of the data accumulated relative to the absorption of the egg yolk shows that 90 per cent of the yolk present at hatching is absorbed during the first 5 days of life. The fat content of the egg yolk

decreased even more rapidly for it dropped from 20.6 per cent in the unabsorbed yolk present in 1-day-old chicks to 13.9 per cent for the unabsorbed yolk present in 5-day-old chicks. The vitamin A units present in the unabsorbed yolk decreased from 49.5 units for the 1-day-old chicks to 22.3 units for the 5-day-old chicks. The combined vitamin A potencies of the liver and the unabsorbed yolk decreased from 65.5 units for the 1-day-old chicks to 51.6 units when the chicks were 3 days old. The chicks were fed at this time.

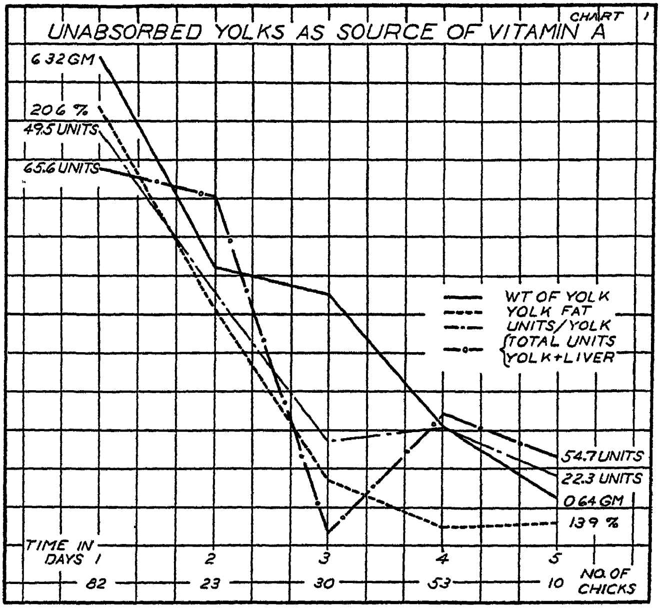

Chart 1

Subsequent to feeding the vitamin A potencies of the unabsorbed yolk and liver increased to 57.4 units when the chicks were 4 days old and then fell to 54.7 units when the chicks were 5 days old.

It is of interest to correlate the rate of growth of young chicks with their vitamin A reserve. Accordingly data in this connection have been assembled from studies of 298 chicks killed at different periods between the first and fourteenth day of age. The average values computed from these data

have been plotted as graphs (chart 2). The average body weight of the chicks at 1 day of age was 40.4 gm., but it decreased during the next 2 days, when feed is normally withheld from baby chicks, to 35 gm. Subsequently the body weight increased rapidly to 98.4 gm. when the chicks were 14 days of age. The average weight of the liver was 1 gm. when the chicks were 1 day old and it increased to 4 gm. when the chicks were 2 weeks old. The fat content of the

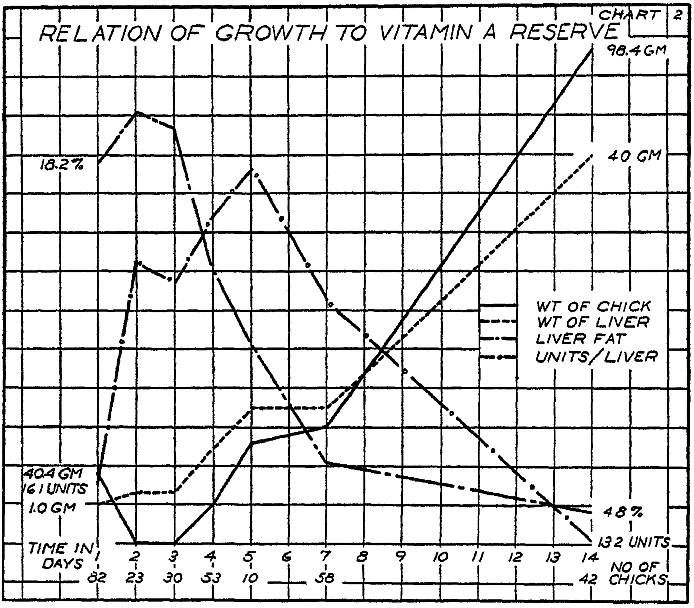

Chart 2

liver decreased from 18.2 per cent for 1-day-old chicks to 4.8 per cent for 14-day-old chicks. The vitamin A potency of the livers increased very rapidly from 16.1 units at 1 day of age to 32.2 units at 5 days of age, while the chicks were utilizing the vitamin A stores of the unabsorbed yolk. After the egg yolk had been absorbed, by the end of the fifth day of life, the vitamin A potency of the chicks' livers decreased rapidly to 13.2 blue units per liver when the chicks were 14 days old.

SUMMARY

Data concerning the vitamin A stores of embryo and young chicks have been accumulated by assaying the livers from 100 chick embryos and 298 young chicks. Since the unabsorbed egg yolk is a rich source of vitamin A these were assayed.

The livers from forty embryo chicks at the eighteenth day of incubation weighed 0.51 gm. and contained 12.5 per cent of fat and 7 blue units of vitamin A. The unabsorbed yolk weighed 14.3 gm. and contained 26.7 per cent fat and 80 blue units.

The livers from thirty 6-hour-old chicks weighed 0.89 gm. contained 17.2 per cent fat and had a reserve of 12 blue units of vitamin A. The corresponding values for the unabsorbed yolk were, weight 8.3 gm., fat 24.2 per cent and 58 blue units of vitamin A.

The average weight of livers from 24-hour-old chicks was 1.10 gm., the fat and vitamin contents were 18.8 per cent and 19 blue units, respectively. The values for the unabsorbed yolks were weight 5.1 gm., fat 18.4 per cent and 44 blue units of vitamin A.

Two lots of forty baby chicks were obtained from two sources. Groups of ten chicks were killed at 24-hour intervals. The body weight of the chicks decreased continuously until feeding was commenced. During the first 4 or 5 days of life the weight of the livers and their vitamin A content increased but the fat content of the livers and the weight, fat and vitamin A content of the unabsorbed yolks decreased. The rapid increase in the vitamin A content of the livers of chicks following hatching is doubtless influenced by the large store of vitamin A present in the egg yolks.

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THE AMINO ACID CONTENT OF EGGS AND CHICKS: RELATION TO DIET AND TO INCIDENCE OF CHONDRODYSTROPHY [1]

A. R. PATTON AND L. S. PALMER
Division of Agricultural Biochemistry, University of Minnesota, St. Paul

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Pollard and Carr ('24) and Gerber and Carr ('30) reported differences in the Van Slyke nitrogen distribution of eggs from pigeons on different diets. McFarlane, Fulmer and Jukes ('30) were unable to find any significant influence of diet on the amino acid content of hen eggs. Titus, Byerly, and Ellis ('33) reported evidence of a slight effect of the diet on the total nitrogen of the egg yolk. Calvery and Titus ('34) were unable to find any marked difference in the composition of the proteins of eggs from pullets on different protein diets.

Titus, Byerly and Ellis also found that certain protein diets tend to lower the hatchability of the eggs produced. Part of the chick mortality was shown to be caused by chondrodystrophy (Chondrodystrophia foetalis), a type of defective bone and cartilage formation of unknown etiology. Their evidence indicated an amino acid deficiency as a possible factor in the etiology of chondrodystrophy. Since this abnormality represents primarily a failure in cartilage development, we observed that gelatin, the best known protein in cartilage, contains about 22 per cent glycine. Corn, on the other hand, contains no glycine in its chief protein, zein.

[1] Paper no. 1382, journal series, Minnesota Agricultural Experiment Station. The data in this paper were submitted by A. Rae Patton to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of doctor of philosophy, June, 1935. All pertinent individual data are available for reference from the Division of Agricultural Biochemistry.

A corn ration was found by Byerly, Titus and Ellis ('33 a, '33 b) to permit an exceedingly high incidence of chondrodystrophy. It is for this reason that the glycine content of eggs was investigated with reference to the incidence of chondrodystrophy.

EXPERIMENTAL

The following rations were used:

Optimum ration

	Per cent
Ground yellow corn	20
Standard wheat middlings	20
Ground oats	20
Bran	10
Meat and bone meal	5
Fish meal	5
Soybean meal	5
Milk	5
Alfalfa leaf meal	6
Wheat germ	3
Cod liver oil	1
NaCl, $CaCO_3$, corn, wheat	ad. lib.

Deficient ration

	Per cent
Ground yellow corn	70
Corn gluten (lot 39)	22
Cod liver oil	1
Wheat germ	3
Oat hulls	1.5
Monosodium phosphate	0.9
Calcium carbonate	1
Sodium chloride	0.5
NaCl, $CaCO_3$, corn	ad. lib.

Fourteen white Leghorn pullets were raised on the 'optimum' ration, and then restricted to the 'deficient' ration from May 4 to August 12, 1933. During this period average egg production dropped from eight eggs per day to less than two eggs per day. Eggs produced on the 'optimum' ration, and others produced near the close of the period on the 'deficient' ration were analyzed. The entire egg contents were extracted 48 hours each with acetone, alcohol, and ether, yielding samples containing 16.0 per cent nitrogen. In the case of chick samples,[2] the entire egg contents were treated in the same manner. A sample for analysis consisted of the contents of six eggs.

Tryptophane and tyrosine were determined by the method described by Folin and Marenzi ('29), histidine by the Rosedale-Da Silva method ('32), total surfur by the Denis method

[2] Chondrodystrophic material was identified and provided by Dr. F. B. Hutt, who also supplied the normal chicks for comparison.

('10). The results are given in table 1. Van Slyke nitrogen distribution was determined according to the Cavett modification ('32). The duplicate analyses are given in table 2. The glycine analyses shown in table 3 were made by the method described by Patton ('35), and the total creatinine by the method of Folin ('14). The glycine data for the egg

TABLE 1

Tryptophane, tyrosine, histidine and total sulfur determinations on eggs from hens fed optimum and deficient (corn) rations

	TRYPTOPHANE	TYROSINE	HISTIDINE	SULFUR
	per cent	*per cent*	*per cent*	*per cent*
Eggs from White Leghorns optimum ration	1.08 ± 0.06	4.27	2.26	1.3
Eggs from same flock after 14 weeks on corn ration	1.06 ± 0.05	4.28	2.35	1.4

TABLE 2

Van Slyke nitrogen distribution. The figures are expressed as percentage of total nitrogen

	EGGS FROM HENS FED				NORMAL CHICKS		CHONDRODYSTROPHIC CHICKS	
	Optimum ration		Deficient ration					
Ammonia	14.62	14.75	10.06	12.50	13.50	13.70	16.60	16.20
Humin	10.00	7.38	8.07	4.75	8.63	8.77	9.46	8.12
Filtrate amino	37.80	38.10	43.30	46.20	39.20	38.40	35.70	36.60
Filtrate non-amino	9.70	10.90	10.90	10.80	13.80	14.10	14.00	14.20
Arginine	9.75	9.75	11.00	11.00	8.77	8.77	8.77	7.80
Cystine	0.39	0.39	0.42	0.33	0.36	0.36	0.41	0.42
Histidine	6.40	6.40	3.90	4.55	6.90	7.60	5.40	6.70
Lysine	9.76	9.76	11.97	11.42	11.37	10.67	9.73	9.38
Total	98.42	97.43	99.62	101.55	102.53	102.37	100.07	99.42

samples represent the average of concordant results from single samples and for the chicks the mean of average data from two samples.

In addition to the samples described, eggs were collected from two white Leghorn pullets fed the optimum ration plus 1 gm. of glycine per day, in capsules. Glycine was determined in the eggs before and after 1 month of glycine feeding.

Glycine exerted toxic action in large doses (4 gm. a day). Two days of this treatment resulted in cessation of egg production. Food and water consumption were practically zero. Both hens exhibited extreme prostration, inability to stand, and sudden fits of agitation. When glycine feeding was discontinued, the symptoms rapidly disappeared, and normal egg production was resumed.

TABLE 3

Glycine and total creatinine determinations

	MEAN GLYCINE CONTENT OF TOTAL 'PROTEIN'	TOTAL CREATININE IN DRY MATTER
	per cent	*per cent*
Eggs from White Leghorns, optimum ration	2.21	
Eggs from same flock after 14 weeks on corn ration (glycine deficient)	2.16	
Eggs from hen R on optimum ration before feeding glycine	1.80	
Eggs from hen R on optimum ration plus glycine	1.87	
Eggs from hen L on optimum ration plus glycine	1.82	
Chick embryos which developed normally	9.5 9.1	0.02
Chick embryos which died from chondrodystrophy	6.8 6.3	0.02

DISCUSSION

On the basis of the glycine determinations certain conclusions may be drawn. In the first place, feeding a low glycine ration does not alter the glycine content of the eggs which the hen lays. Also, feeding a high glycine ration does not alter the glycine content of the eggs, either free or combined in protein. Since incubated eggs contain more glycine than fresh eggs, there appears to be a synthesis of glycine during embryonic development.

It is interesting that the total creatinine values found were the same in both normal and chondrodystrophic chick samples. This may constitute evidence of the similarity in age of the

chick groups compared, since total creatinine is known to increase rapidly during incubation (Needham, '31).

Any comparisons between the composition of the rations and of the chicks shown in table 2 would be purely theoretical, since the chondrodystrophic chicks were not produced on the 'deficient' ration used. The ammonia nitrogen values diverge in an unexpected fashion, as do the humin, filtrate amino, and lysine nitrogen values. The significance of this cannot be interpreted at the present time. Arrangements have been made for continuing the phase of this work concerning amino acids and chondrodystrophy.

SUMMARY

1. A significant difference was found between the glycine contents of normal and chondrodystrophic embryos.

2. A synthesis of glycine during development of the hen's egg was demonstrated.

3. Glycine was found to be toxic to hens when fed in large doses.

4. Feeding glycine to hens did not influence the glycine content of the eggs.

5. An investigation has been made of the amino acid content of eggs from fowls receiving 'optimum' and 'deficient' protein rations.

6. The analyses, including tryptophane, tyrosine, histidine, glycine, arginine, cystine, lysine and the Van Slyke nitrogen distribution, did not demonstrate a significant effect of the diet on the composition of the egg proteins.

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ERRATUM

Volume 11, page 135. Footnote 1 should read as follows:

Working under a grant from the California Fruit Growers Exchange.

THE EXCRETION OF VITAMIN C IN NORMAL INDIVIDUALS FOLLOWING A COMPARABLE QUANTITATIVE ADMINISTRATION IN THE FORM OF ORANGE JUICE, CEVITAMIC ACID BY MOUTH AND CEVITAMIC ACID INTRAVENOUSLY

ESTELLE E. HAWLEY,[1] D. J. STEPHENS AND GEORGE ANDERSON[2]

Departments of Pediatrics, Medicine and Physiology, University of Rochester, School of Medicine and Dentistry, Rochester

FOUR FIGURES

(Received for publication October 2, 1935)

The application of the titration method to the estimation of vitamin C in urine has resulted in the recent suggestion that the urinary excretion of vitamin C can be used as a diagnostic test of vitamin C subnutrition in humans. Harris and Ray ('35) found that normal adults, on average diet, excreted from 15 to 30 mg. of vitamin C daily. The administration of large amounts of orange juice to such individuals resulted in a prompt and marked increase in the urinary output (Harris, Ray and Ward, '33; Hess and Benjamin, '34; Johnson and Zilva, '34). On the other hand, when the test dose was administered to patients with scurvy, or to those with a history of vitamin C underfeeding, the urinary excretion remained low. As a result of these observations it was suggested that the urinary excretion of ingested vitamin C was an index of the degree of saturation of the individual and therefore might be used as a test for hypovitaminosis C. Others have in general confirmed these observations but have reported a low urinary output after the test dose in some

[1] Working under a grant from the California Fruit Growers Association.

[2] Supported by the University of Rochester Grant for Fluid Research.

normal persons (Schröder, '35; Youmans, Corlette, Akeroyd and Frank, '35), in infectious diseases (Schröder, '35) and in some vascular disorders (Finkle, '35). Before such a test may be applied to clinical use, the range of physiologic variation in normal subjects, the effect of varying degrees of vitamin deprivation and the influence of various pathologic states on vitamin C excretion must be experimentally determined.

In this investigation the effect of variations in the intake of vitamin C on the urinary excretion has been studied under controlled conditions in a group of twelve normal adults. The 24-hour excretion was determined during a period of low vitamin C intake, and also during the daily administration of large amounts of vitamin C in the form of orange juice. After saturation, the effect of comparable amounts of vitamin C in the form of orange juice and of crystalline cevitamic acid ('Cebione' Merck & Co.) by mouth and of the latter intravenously was observed. In addition, the vitamin C content of whole blood was determined at appropriate intervals.

MATERIALS AND METHODS

Four female and eight male subjects were studied. Two of the women (L., J.C.) were graduate nurses, one (Je.C.) was a medical student and one (A.M.) a technician. Seven of the men were medical students, the other one a hospital interne. The age of the subjects varied between 21 and 30 years. With the exception of L.K., all were in excellent health and habitually ingested diets containing at least average amounts of antiscorbutic foods. L.K. gave a history of gingival bleeding for several months and of low dietary intake of vitamin C.

The first 24-hour urine sample was collected while the subject was taking his usual diet. During the following week the diet remained as before, except that all fresh fruits, vegetables, and tomatoes or tomato juice were eliminated. It was estimated that such restriction in the diet reduced the daily intake of vitamin C to a low level (10 to 20 mg.). This basal diet was continued throughout the remainder of the experiment except as noted below. During the second period of

8 days, 400 cc. of orange juice were given in a single dose each day after breakfast. During the third period the daily ration of orange juice was reduced to 200 cc. The orange juice was freshly squeezed from California oranges and contained 50 to 60 mg. of cevitamic acid per 100 cc., as determined by the titration method on a number of occasions. The value of 50 mg. per 100 cc. orange juice was used since the error in using this approximate value was no greater than that which is normally introduced in such dietary experiments. At the end of the third period 100 mg. of cevitamic acid were given first orally and then intravenously on successive days. Blood samples were taken for the determination of vitamin C content at the beginning of the first and second periods and at the end of the third period. At the conclusion of the experiment, four individuals took a vitamin C-free diet (meat, bread, cereals, pasteurized milk) for 2 days, and on the third day in addition received a large portion of orange juice (400 to 1000 cc.). Temperature and weight were recorded daily. No attempt was made to regulate activity; however, variations from the normal routine of any subject were recorded for reference.

Twenty-four-hour urine samples were collected from 7 A.M. to 7 A.M. daily. Each individual specimen was placed, as soon as voided, in a bottle containing glacial acetic acid to insure against loss of vitamin C. When not in use the bottle was kept in a cold, dark place. Incomplete 24-hour samples were discarded. Vitamin C content was determined by rapid titration of the urine against a measured volume of a standardized solution of 2:6-dichlorophenolindophenol, according to the method described in detail by Harris and Ray ('35). The requirements which Ahmah ('35) suggests are necessary for accuracy in the determination were met in these titrations. Determination of blood vitamin C were made in trichloroacetic acid filtrates of oxalated blood.

Capillary fragility determinations were made routinely each day by the method described by Dalldorf ('33).

RESULTS

During the initial period of curtailed vitamin C intake, several of the subjects complained of anorexia, fatigue and irritability. In the majority of instances these complaints were made voluntarily. Without exception, they disappeared within a few days after the administration of orange juice was begun. L.K. noted increased gingival bleeding during the period of vitamin C limitation but within a few days after taking large amounts of orange juice, bleeding from the gums entirely disappeared for the first time in several months. It is of interest that this subject, as indicated in table 2, had the lowest concentration of vitamin C in his blood in the beginning of the experiment and had the second highest value at the end of the high C diet. Inspection of the record of the urinary excretion shows the ease with which his tissues became saturated. Otherwise each subject remained in good health throughout the experimental period. No significant change was observed in the temperature or weight.

Estimations of the intake and 24-hour excretion of vitamin C in each of the twelve subjects are shown in figures 1, 2 and 3. Twenty-four-hour excretion while on a normal diet varied between 15 and 28 mg. The majority of the subjects continued to excrete vitamin C in approximately the same amount during the period of limited vitamin C intake. Two individuals (Je.C. and J.C.) excreted smaller amounts during this period.

Considerable variation was observed in the response to the addition of orange juice. G.A. and J.C. showed significant increases in urinary output on the first day. R.A., on the other hand, continued to excrete only small amounts of cevitamic acid until the ninth day, when 45 per cent of the test dose appeared in the urine. In the other subjects the urinary content of vitamin C began to rise on the second, third or fourth day after the addition of orange juice. Fluctuations also occurred after saturation had presumably been accomplished. A few individuals (G.A., A.M. and L.K. in particular) consistently excreted large amounts of the test dose each

day. Others showed striking and unexplained variations. In several subjects (G.A., L., A.M., J.F. and L.T.) the 24-hour excretion exceeded 100 per cent the daily test dose within a few days after the administration of orange juice was begun. The same test dose on subsequent days, however, resulted in

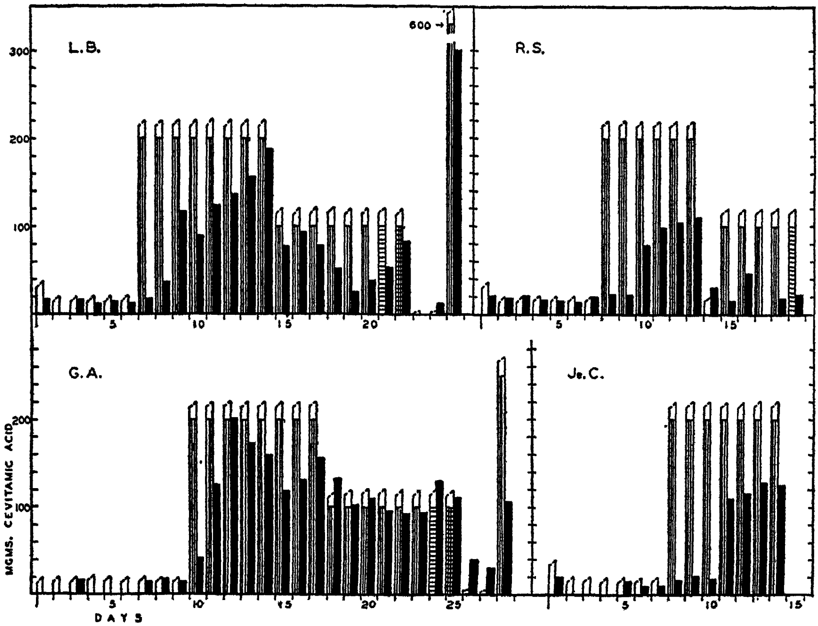

Fig. 1 The daily intake and 24-hour excretion of cevitamic acid. Open columns indicate estimations of vitamin C in the diet. Columns with vertical lines indicate vitamin C administered as orange juice. Columns with horizontal lines indicate vitamin C given as cevitamic acid by mouth, cross-hatched columns, cevitamic acid intravenously. The 24-hour excretion of vitamin C in the urine is represented by the solid columns. Blank spaces for the latter indicate that the 24-hour specimen was incomplete and therefore discarded.

the elimination of smaller amounts, which, in several instances, were but little above the control level. Reduction in the size of the test dose resulted in a roughly comparable decrease in excretion.

In table 1 are summarized the results of the administration of orange juice and of cevitamic acid by mouth and intravenously. Although here again, considerable individual variation

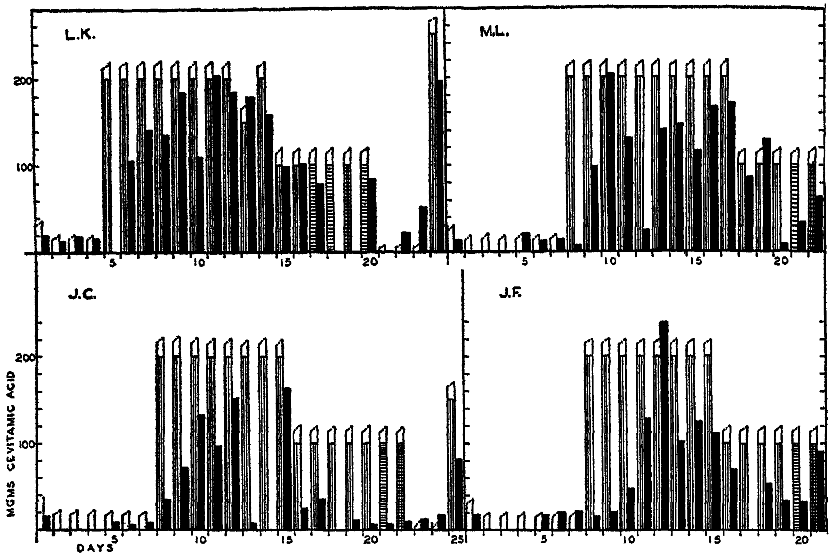

Fig. 2 Legend as in figure 1.

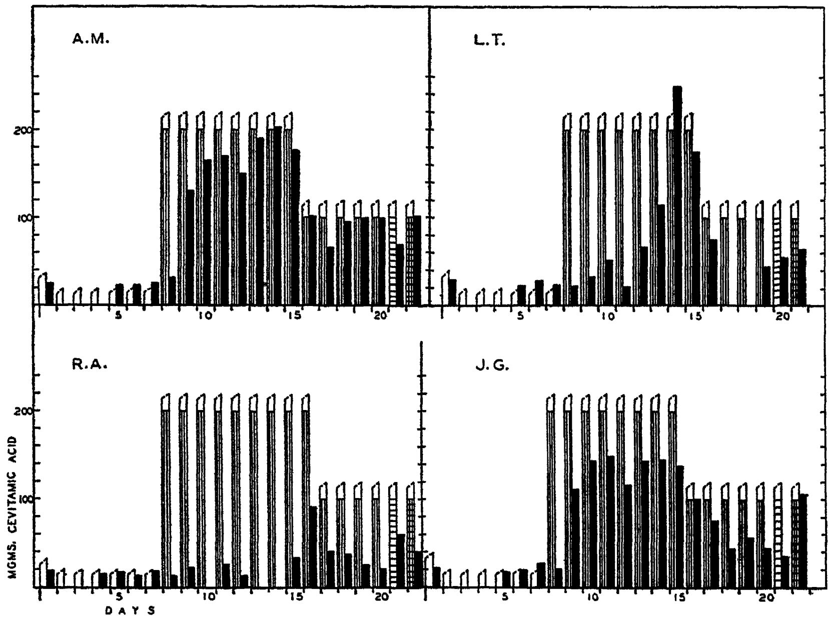

Fig. 3 Legend as in figure 1.

was observed, the averages show that approximately the same proportion of the vitamin is excreted when given in equivalent amounts as orange juice and as crystalline cevitamic acid. The urinary output is almost doubled, however, by intravenous administration.

In three individuals the hourly excretion of the test dose was determined before and after saturation. The results of these observations are shown in figure 4. The initial determination in each instance was made immediately after the control period, with the first of the daily doses of 400 cc. of

TABLE 1

Twenty-four-hour excretion of cevitamic acid in response to test doses of vitamin C after saturation with orange juice

SUBJECT	200 CC. ORANGE JUICE (APPROXIMATELY 100 MG. CEVITAMIC ACID)	100 MG. CEVITAMIC ACID BY MOUTH	100 MG. CEVITAMIC ACID INTRAVENOUSLY
	mg.	*mg.*	*mg.*
G.A.	93.4	129.2	110.9
R.A.	19.6	60.4	40.2
L.B.	51.0	52.0	82.7
J.C.	6.4	5.7	9.6
F.	33.0	...	91.5
J.G.	44.1	34.0	105.00
L.	8.8	34.5	63.8
A.M.	98.0	69.5	102.0
S.	18.2	22.6	....
L.T.	43.5	54.3	63.8
Average	41.6	41.3	73.3

orange juice, representing approximately 200 mg. of vitamin C. Subsequent determinations after saturation were made following the administration of 100 mg. of vitamin C given, usually on successive days, as orange juice and as crystalline cevitamic acid by mouth and intravenously. When orange juice and cevitamic acid were taken by mouth, the urinary content of vitamin C began to increase during the first hour and reached a peak in from 3 to 6 hours. After intravenous administration, however, a large proportion of the test dose was excreted in the first hour. Significant differences in the excretion curves before and after saturation

were apparent despite the fact that the initial dose was twice the size of those employed later. As might be expected, the introduction of the material directly into the blood stream resulted in its excretion in the urine more quickly and more completely than when taken by mouth.

The results of withdrawal of vitamin C in four individuals after saturation are included in figure 1. The urinary excretion of three subjects dropped promptly to the control level;

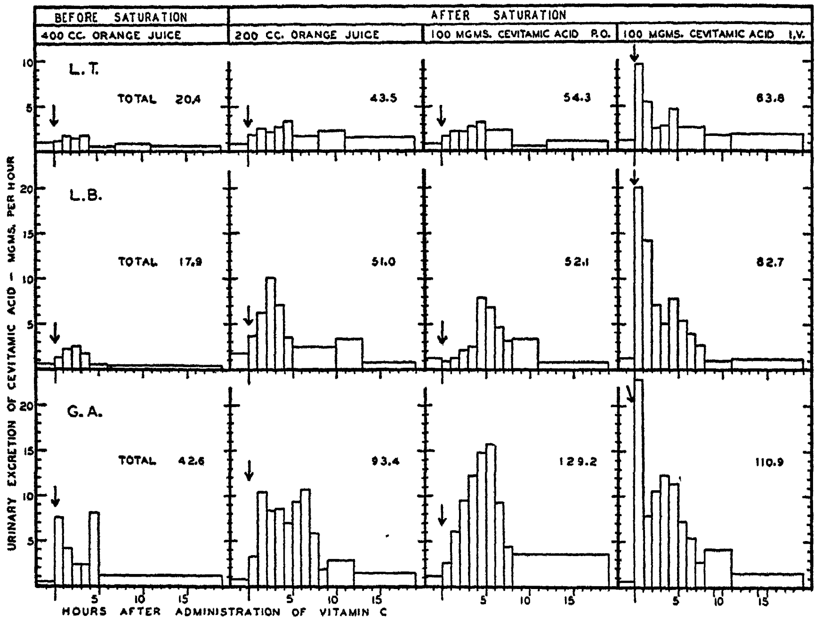

Fig. 4 The hourly excretion of test doses of vitamin C before and after saturation with orange juice. Arrow indicates time of vitamin C administration.

the fourth excreted amounts somewhat above those observed during the control period. In each instance, the administration of a large amount of orange juice after 2 days of strict vitamin C deprivation resulted in the excretion of a large proportion of the test dose.

Determinations of the vitamin C content of whole blood are shown in table 2. Although in several cases higher values were obtained after saturation, the differences are small and

probably without significance. These figures are, on the whole, slightly lower than those recently reported in a large series of individuals by Mirsky, Swadesh and Soskin ('35) who likewise observed no correlation between the ascorbic (cevitamic) acid content of blood and the dietary regime.

No significant changes were noted in capillary fragility. Detailed results of this phase of the experiment will be reported in a later paper.

TABLE 2

Whole blood content of vitamin C

SUBJECT	BEGINNING OF PERIOD 1 (NORMAL DIET)	BEGINNING OF PERIOD 2 (AFTER LOW-C DIET)	END OF PERIOD 3 (AFTER HIGH-C DIET)
	mg. per cent	*mg. per cent*	*mg. per cent*
G.A.	0.69	0.81	1.21
A.	1.32	1.00	1.15
L.B.	1.00	0.93	1.63
Je.C.	1.02	0.88	...
J.C.	1.19	1.72	1.25
F.	0.93	0.83	1.00
J.G.	0.95	1.19	0.86
L.K.	0.58	1.16	1.47
L.	0.92	0.96	1.40
A.M.	0.94	0.83	0.85
R.S.	1.07	0.91	...
L.T.	0.98	0.80	1.10
Average	0.96±0.037	1.00±0.047	1.21±0.051

Difference 1 and 2 = 0.04±0.063.
Difference 1 and 3 = 0.25±0.064.
Difference 2 and 3 = 0.21±0.066.

DISCUSSION

In several respects the observations here reported confirm those previously recorded by other investigators. The values for the urinary excretion of vitamin C by normal individuals on an average diet compare favorably with those reported by Harris and Ray ('35) in England, by Schröder ('35) in Germany and by Youmans, Corlette, Akeroyd and Frank ('35) in this country. We are also able to confirm the observation (Harris and Ray, '35) that in the majority of individuals the normal amounts of vitamin C continue to be

excreted for at least several days after the dietary intake of antiscorbutic foods has been curtailed. A delay of from 1 to several days in the excretion of a daily test dose of vitamin C was observed in individuals whose tissues had presumably become 'unsaturated' by a short period of vitamin deprivation. This observation apparently supports the contention that the reserve stores of such individuals are quickly depleted and must be repleted before the ingested vitamin C appears in more than normal amounts in the urine.

It is noteworthy that considerable variation was observed in the time required for increased urinary excretion to begin after the addition of orange juice to the diet. Such variation might be expected if the control period were of insufficient duration to reduce the subject to a common level of unsaturation. On the other hand, it is possible that individuals differ in the efficiency with which vitamin C is absorbed, stored and excreted.

After saturation had presumably been accomplished, wide fluctuations were observed in the urinary response to repeated daily test doses.

These observations suggest that factors other than the 'state of saturation' of an individual may influence the urinary excretion of vitamin C after a given test dose of orange juice.

Further studies may reveal hitherto unrecognized pitfalls in the method used in determining the vitamin C content of urine, or factors which have not been satisfactorily controlled in these experiments; a larger group of normals might show a decreased percentage of variability. Unless the variations in the response of apparently normal individuals can be predicted or controlled, they offer a serious limitation to the clinical use of the test suggested for the diagnosis of subclinical vitamin C deficiency states.

SUMMARY

1. The effect of variations in the daily intake of vitamin C on the urinary excretion of cevitamic acid has been studied in twelve normal young adults.

2. The urinary excretion of vitamin C by individuals on an average normal diet varied between 15 and 28 mg. per 24 hours.

3. Excretion continued at a steady rate during a preliminary control period of low vitamin C intake.

4. Considerable individual variation was observed in the urinary response to repeated test doses of orange juice, both during and after apparent saturation with vitamin C.

5. Comparable amounts of vitamin C given orally as orange juice and as cevitamic acid resulted in similar urinary excretion curves. Cevitamic acid administered intravenously was excreted more rapidly and more completely than when given by mouth.

6. Variations in the intake of vitamin C had no demonstrable effect on the cevitamic acid content of whole blood, or on the capillary fragility.

The authors wish to express their appreciation to Dr. S. W. Clausen for his interest and helpful suggestions.

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THE ROLE OF CALCIUM AND PHOSPHORUS IN DETERMINING REPRODUCTIVE SUCCESS

WARREN M. COX, JR. AND MIRIAM IMBODEN
Research Laboratory, Mead Johnson & Co., Evansville, Indiana

SEVEN TEXT FIGURES AND ONE PLATE

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Many factors influence successful gestation and lactation: one of these is the calcium and phosphorus content of the diet. Deficiencies of these elements are not as immediately apparent as are deficiencies in dietary organic constituents (Meigs, '22), but usually appear only after several reproductive cycles. Simmonds ('24) observed that on a diet deficient in calcium (0.103 per cent) rats were able successfully to suckle as many as two litters of young but that they subsequently failed even to reproduce.

Most reports in this field have embraced but a short span of the life cycle of the experimental animal: the period of growth, or fractions thereof; isolated observations throughout gestation; or, the period of lactation. In view of the relative incompleteness of short periods of observation we thought it desirable to study the behavior of experimental animals receiving constant amounts of dietary calcium and phosphorus throughout the entire span of their reproductive life. We hoped that such life-time observations would enable us to draw definite conclusions regarding the optimal calcium-phosphorus relationship in pregnancy and lactation.

LITERATURE

The observation quoted above (Simmonds, '24) is the only report we have found regarding the specific type of problem in which we were interested, although Hunscher ('30) studied

calcium balances for two successive lactation periods in women. Sherman and Muhlfeld ('22) have studied reproductive behavior on certain milk diets, but their diets involved changes in many variables.

Many investigators have reported optimal Ca : P ratios, although Sherman ('32 a) warns against such statements without exhaustive research, his thought being that the optimal ratio will change throughout the different life stages, and from species to species. Usually such ratios have been based on growth curves (Bethke, Kick and Wilder, '32), on blood serum values (Kramer and Howland, '32), or on bone-ash determinations (Brown, Shohl, Chapman, Rose and Saurwein, '32) in the rat, although calcium balances in the lactating cow (Ellenberger, Newlander and Jones, '32), and goat (Hart, Steenbock, Kletzien and Scott, '26–'27) have been widely employed. Optimal ratios for the chick (Wilgus, '31) have been determined by measuring growth and bone ash. The effect of different ratios on the strength of bone of growing pigs was studied by Bethke, Edington and Kick ('33). Such investigations have in general indicated Ca : P ratios between 1.0 and 2.0 as optimal. In recent years the relation of calcium and phosphorus to rickets has received much attention (Bethke, Kick and Wilder, '32; Kramer and Howland, '32; Brown, Shohl, Chapman, Rose and Saurwein, '32).

Based upon the mineral retentions of infants and children, Stearns ('31) considers a Ca : P retention of less than 1.5 : 1.0 indicative of rapid muscular or tissue growth, and a retention ratio of 2.0 indicative that the majority of retained mineral elements are used to form bone. In early infancy she finds the ratio of retention is less than 1.5, but that in childhood it approaches 2.0.

During lactation, retention of calcium and phosphorus is admittedly poor. Metabolism studies with the lactating cow and nursing woman (Donelson, Nims, Hunscher and Macy, '31) emphasize the strain on maternal mineral reserves during this period. The first part of lactation was usually characterized by negative calcium balances, and only when milk flow diminished was calcium stored. Additional vitamin D

during this first period may (Hart, Steenbock, Kletzien and Scott, '26–'27), or may not (Hart, Steenbock, Teut and Humphrey, '29) change a negative to a positive balance, although it usually improves the balance. The literature on this subject has been reviewed by Macy, Hunscher, McCosh and Nims ('30).

Negative calcium balances are commonly observed during gestation. Toverud and Toverud ('31) obtained forty-four balances on sixteen expectant mothers receiving the ordinary institutional diet, occasionally supplemented. The following table is compiled from their findings.

Calcium and phosphorus balances at different levels of intake compiled from the data of Toverud and Toverud ('31)

LEVEL OF INTAKE		AVERAGE INTAKE	NUMBER METABOLISM PERIODS	AVERAGE MONTH OF PREGNANCY	NEGATIVE BALANCES, PER CENT
Calcium	Less than 1 gm.	0.83	17	7.6	41.2
	More than 1 gm.	1.35	27	7.6	14.8
Phosphorus	Less than 1 gm.	0.82	6	7.0	83.3
	More than 1 gm.	1.48	38	7.7	13.2

Their results show, fairly definitely, that when the mineral intake is above 1.0 gm. daily, positive balances may be expected, but that intakes below this level predispose to negative balances. They feel that the level of mineral intake is a more important factor in obtaining positive balances than the presence of vitamin D. On the other hand, Macy, Hunscher, Nims and McCosh ('30), frequently obtained negative balances with calcium intakes considerably greater than 1.0 gm. The adult maintenance allowance of Sherman ('32 b) (0.68 gm.) is evidently insufficient for periods of mineral stress. The fact that frequently retention during pregnancy does not meet the mineral requirement of the fetus has been emphasized (Coons and Blunt, '30).

The foregoing incomplete survey may be summarized from a nutritional standpoint as follows:

1. Optimal Ca:P ratios, for various species, have been provisionally established at 1.0 to 2.0, although Sherman's ratio is 0.5 ('32 b).

2. Gestation is frequently accompanied by negative calcium balances. Where positive balances are obtained, retention is frequently less than calculated fetal requirement.

3. The level of mineral and vitamin D intake are of importance.

4. Early lactation is accompanied by negative mineral balances in spite of adequate intake. Vitamin D may be of value during this period. Late lactation, when milk flow has declined, is the period of storage.

5. The only evidence adduced for a change in optimal ratios of intake during different life periods is from retention data. As yet, no important species difference has been reported.

6. Continued low calcium intake may result in reproductive failure.

EXPERIMENTAL

One hundred and forty-five virgin rats, carefully selected from our stock colony,[1] were mated and allowed to raise their first litter while receiving stock food (Bills' modification of the Steenbock diet).[2] The technic employed with this control litter was routinized, and employed for ten subsequent reproductive cycles on experimental diets, litters were reduced to six pups at birth, the young were killed and ashed at 21 days of age, and the mother was remated on the day of weaning.

After successfully raising their first litters on stock food, five mothers were placed on each experimental diet, and their average performance during ten subsequent cycles taken as an expression of the suitability of a given calcium-phosphorus level and ratio for gestation and lactation. In order to evaluate performance three criteria were chosen: 1) the average weight of the young at 21 days; 2) their percentage ash; and 3) the change in weight of the mother throughout

[1] Rats weighing 190 to 230 gm. (average 199.4), at 79 to 127 days (average 99) were selected. They were considered unsuitable if 1) they did not become pregnant after one mating; 2) if they gave birth to less than six young; 3) if they did not raise young to 21 days of age; 4) if the young weighed less than an average 25 gm. at 21 days; and 5) if the mother failed to gain weight.

[2] Yellow maize, 57.0; whole milk powder, 25.0; linseed oil meal, 12.0; crude casein, 3.7; alfalfa leaf meal, 1.5; iodized table salt, 0.4; calcium carbonate, 0.4.

the cycle. For the purpose of grading relative performance ten mothers were carried through the entire eleven cycles on stock food.

Basal diet. The basal diet consisted of:

Casein (acid washed)	20.0
Dextrinized starch	50.1–55.1
Lard (Swift's Silverleaf)	9.0
Yeast concentrate (equivalent in B to 24 per cent, in G to 8 per cent, yeast)	4.0
Wheat germ oil	1.6
Carotene solution in oil 3 : 1000	0.3
Salts—Ca and P free[3]	3.1
Rice cellulose	1.2– 5.9

This diet contained 0.018 per cent calcium and 0.245 per cent phosphorus. No vitamin D was added, and the animals were housed in a room protected from ultraviolet light. Two-kilogram batches of each diet were mixed not less often than every 2 weeks, and, when not in use, stored at 10°C. Record was made of individual food and water consumption.

Calcium-phosphorus mixtures. To the basal diet predetermined quantities of dicalcium phosphate and either monobasic ammonium phosphate or calcium acetate, were added. The maximum amount of dicalcium phosphate was used, and the additional required element made up by one of the other two salts. The dietary levels of the two elements are indicated in the following table, together with the diet number and Ca : P ratio.

TABLE 1

Levels and ratios of calcium and phosphorus employed

PERCENTAGE PHOSPHORUS IN DIET	PERCENTAGE CALCIUM IN DIET									
	0.245		0.490		0.735		1.225		2.450	
	Diet No.	Ca/P Ratio	Diet No.	Ca/P Ratio	Diet No.	Ca/P Ratio	Diet No.	Ca/P Ratio	Diet No.	Ca/P Ratio
0.245	1	1.0	6	2.0	11	3.0	16	5.0	21	10.0
0.490	2	0.5	7	1.0	12	1.5	17	2.5	22	5.0
0.735	3	0.33	8	0.66	13	1.0	18	1.66	23	3.33
1.225	4	0.2	9	0.4	14	0.6	19	1.0	24	2.0
2.450	5	0.1	10	0.2	15	0.3	20	0.5	25	1.0

[3] Composition: NaCl 24.50, $MgSO_4$ 9.14, $KHCO_3$ 59.20, KCl 3.84, Fe citrate 2.94, $CuSO_4$ 0.32, $MnSO_4$ 0.04, KI 0.02 per cent.

For completeness, two additional diets were included: Diet no. 26, (the basal ration unsupplemented), and diet no. 27, containing 0.122 per cent calcium and 0.245 per cent phosphorus. (Results with these two diets are reported in tables 2 and 6, and figure 4).

Controls. Two groups of five rats each were used as controls (stock diet). One group was kept in the stock breeding room under conditions that prevail for our rat colony (sterilized wood shavings for bedding and tap water in the drinking bottles). The other group was kept in the experimental room on wire screens—given distilled water to drink, and stock food from our standard iron-plate cup. The laboratory was kept at constant temperature and humidity.

Evaluation of results. For convenience in presenting the data, we have expressed our findings on experimental diets in terms of their percentage relation to the performance of the controls.

1. The weight of young at 21 days of age was converted to a percentage figure as follows:

$$\frac{\text{Average weight experimental young at 21 days}^{4}}{\text{Average weight stock control young in corresponding gestation}} \times 100 \qquad (1)$$

2. Numerous analyses of stock young, 21 days of age, gave an average ash content of 2.775 per cent[5] (Cox and Imboden, '36). It was our original thought that this figure would remain fairly constant for the stock controls, and be independent of the cycle number. By definition 2.775 per cent ash was perfect performance (100 per cent). The lowest percentage body ash we were able to produce after 24 days on McCollum's rachitic diet no. 3143 was 2.000. Regarding this as zero, the

[4] In arriving at the average weight, the number of young that should have been raised (the number born, or a maximum of six) was employed. This procedure was likewise used in computing the average weight of the controls, i.e., 10 × 6 rats should have been raised, during each cycle, by the controls.

[5] Made on the basis of gross dead weight. Before ashing, the gastro-intestinal tract was removed. The carcasses were placed in weighed silica dishes and ashed in an automatically controlled muffle at 550°C.

ash content of the experimental young was converted to a percentage figure as follows.

$$\frac{0.775 - (+2.775 - x)}{0.775} \times 100 \qquad (2)$$

when x is the observed percentage body ash. When x is greater than 2.775 all indicated signs are reversed.

3. The net change in the weights of the mothers during the cycle was converted to a percentage basis, as follows:

$$100 - ([+x - y] \times \tfrac{1}{2})^{6} \qquad (3)$$

when x is the average change in weight of the controls in the particular cycle, and y is the change in weight of the experimental mothers. When y is greater than x all indicated signs are reversed.

The sum of the three individual grades of the five mothers in each group, divided by fifteen gave the net grade for the cycle. The average of ten cycle grades gave the 'success' rating of the diet. When cessation of reproductive activity, or death, occurred, grades of zero were given in subsequent cycles.

RESULTS

We present in table 2 the composite results obtained in this investigation. The compilation permits comparison of any two diets but is not suitable for analysis of the experiment as a whole. Diet no. 13 has been omitted because of the obvious unsuitability of the mothers.

Final success rating. In figure 1 we have plotted the average success rating for the ten cycles (three factors evaluated) against the Ca/P ratio of the diet. There are five curves, indicating five levels of calcium intake. The ratings of the controls are indicated by 'X.'[7]

[6] '½' was included in this formula through the following argument: a 300 gm. rat can lose approximately 66 per cent of its weight before death. If the maximum possible loss (200 gm.) is assumed zero, and the net average gain of the controls assumed 100, 1 gm. gain or loss can be evaluated as ½ per cent.

[7] The grades are less than 100 because of death of the mothers, and a lowered percentage ash of their young.

TABLE 2

The effect of different amounts of dietary calcium and phosphorus on reproduction and lactation

DIET				GESTATION					LACTATION			FACTORS EVALUATED			FINAL 'SUCCESS' RATING	AVERAGE DURATION EXPERIMENT, DAYS	CLINICAL TUMORS	BONE ASH OF MOTHERS	FOOD AND WATER INTAKE			
																			Gestation		Lactation: Increase per pup	
Diet no.	Per cent Ca	Per cent P	Ca/P ratio	Per cent fertile matings	Number completed	Number incomplete	Total living young	Number young available to raise	Number completed	Number young raised	Per cent available young raised	Average weight young at 21 days	Ash content grade	Change in weight of mother					Calories per rat per day	Water cubic centimeters per day	Calories per day	Water cubic centimeters per day
1	0.245	0.245	1.0	90.2	36	1	292	201	35	190	94.5	42.23	90.4	+46	76.0	435	2	61.88	63.9	29.4	8.5	3.9
2	0.245	0.490	0.5	84.9	42	3	302	220	33	156	70.9	43.32	75.7	+36	79.9	540	2	63.58	66.7	31.5	8.7	3.9
3	0.245	0.735	0.33	89.6	43	0	271	222	41	192	86.5	41.42	77.9	+86	93.6	536	4	62.92	62.3	29.4	7.0	3.7
4	0.245	1.225	0.2	80.4	38	3	293	213	36	188	88.3	41.79	84.6	+57	83.5	542	1?	65.98	59.9	40.1	7.7	5.0
5	0.245	2.450	0.1	65.0	12	1	90	68	2	8	11.8	16.60	142.7	—34	10.3	197	0	64.84	53.0	65.9	4.7	...
6	0.490	0.245	2.0	89.6	43	0	330	230	40	206	89.6	43.30	72.1	+55	90.4	527	1	64.56	65.1	33.3	9.7	3.9
7	0.490	0.490	1.0	76.8	42	1	314	229	38	188	82.1	46.66	93.7	+71	95.8	497	2	65.18	59.5	31.8	9.9	3.2
8	0.490	0.735	0.66	79.2	38	4	278	186	28	112	60.2	43.68	86.0	+84	67.3	472	3	66.12	63.5	30.3	8.6	2.9
9	0.490	1.225	0.4	83.7	36	0	315	206	31	146	70.9	41.29	89.4	+74	69.9	481	2	63.92	63.9	34.7	7.5	4.6
10	0.490	2.450	0.2	71.4	23	2	192	131	19	88	67.2	22.54	120.7	—92	35.2	341	0	62.99	57.7	58.6	6.6	3.6
11	0.735	0.245	3.0	72.9	42	1	292	209	36	163	77.5	32.34	69.6	+50	72.8	522	2	64.68	61.5	33.0	8.3	2.6
12	0.735	0.490	1.5	84.6	44	0	319	237	35	179	75.5	44.43	105.7	+58	93.9	493	2	65.82	66.7	36.1	8.5	2.8
14	0.735	1.225	0.6	83.0	38	1	299	214	37	182	85.0	42.68	93.0	+55	83.8	472	1	63.67	59.5	34.2	8.9	5.0
15	0.735	2.450	0.3	78.6	22	0	164	119	15	55	46.2	24.14	85.8	—83	26.8	333	1	61.28	54.0	59.9	4.5	2.6
16	1.225	0.245	5.0	70.6	12	0	94	68	10	51	75.0	21.39	59.2	—32	13.2	381	0	61.83	62.7	30.3	5.1	2.7
17	1.225	0.490	2.5	86.7	38	1	311	211	36	188	89.1	33.74	75.3	+53	73.1	471	1	63.70	58.7	32.4	7.2	3.8
18	1.225	0.735	1.66	90.9	39	1	294	216	35	159	73.6	45.35	98.1	+61	83.4	450	2	65.35	56.3	27.6	10.2	3.4
19	1.225	1.225	1.0	82.4	40	2	318	217	29	141	65.0	42.52	107.1	+105	77.8	431	1?	65.61	61.9	28.6	8.5	3.0
20	1.225	2.450	0.5	76.9	30	0	242	166	24	104	62.7	32.50	102.2	—50	46.4	350	0	57.08	55.8	54.6	6.5	4.2
21	2.450	0.245	10.0	....	2	0	19	12	0	0	0.0	....	....	—126	0.5	185	0	59.40	40.5	22.6	....	...
22	2.450	0.490	5.0	....	2	0	20	12	0	0	0.0	....	....	—40	0.8	351	0	61.36	34.4	21.1	....	...
23	2.450	0.735	3.33	62.1	18	0	124	95	12	57	60.0	19.16	85.7	—42	20.7	362	0	62.60	58.4	40.3	7.7	3.0
24	2.450	1.225	2.0	62.5	21	4	185	119	21	110	92.4	37.33	99.2	+31	40.2	365	1	65.22	61.4	30.2	8.8	3.4
25	2.450	2.450	1.0	90.0	35	1	295	193	19	82	42.5	33.51	105.1	+22	52.8	383	0	63.23	62.5	39.4	6.9	3.4
26	0.017	0.245	0.07	50.0	13	4	99	69	5	22	31.9	18.87	92.7	+5	15.0	261	0	57.77	53.1	28.3	5.3	...
27	0.122	0.245	0.5	68.4	25	1	197	137	17	80	58.4	31.59	92.9	+7	38.1	359	3	61.51	61.1	30.2	7.6	2.7
Stock[1]	0.51	0.47	1.1	79.6	39	0	357	231	38	213	92.2	48.20	92.5	+64	86.1	507	1	63.80	71.6	....	12.2	...
Stock[2]	0.51	0.47	1.1	87.5	42	0	338	234	39	185	79.1	48.73	81.5	+50	91.9	430	0	64.46	66.1	31.9	11.0	3.2

[1] In colony breeding room.
[2] In experimental room.

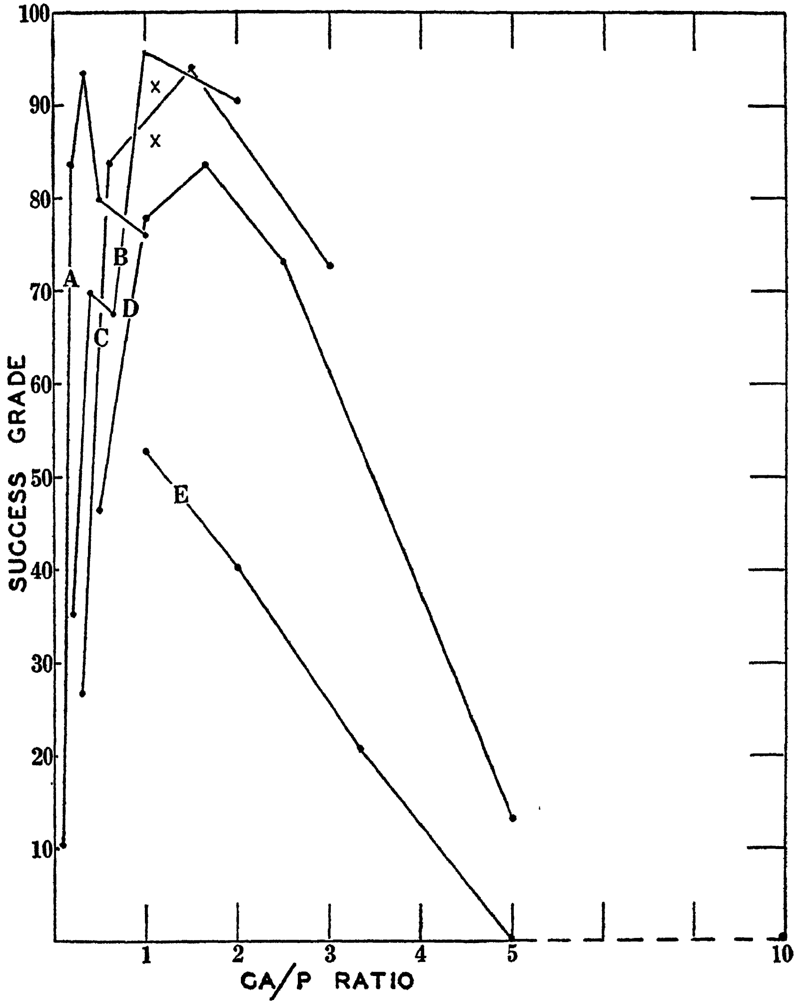

Fig. 1 The 'success' grade of groups of mother rats receiving various levels and ratios of calcium and phosphorus, against the Ca/P ratio of the diet. Levels of calcium are indiated by letters: Curve A, 0.245 per cent level; B, 0.490 per cent; C, 0.735 per cent; D, 1.225 per cent; and E, 2.45 per cent. The grade of the controls is indicated by 'X.'

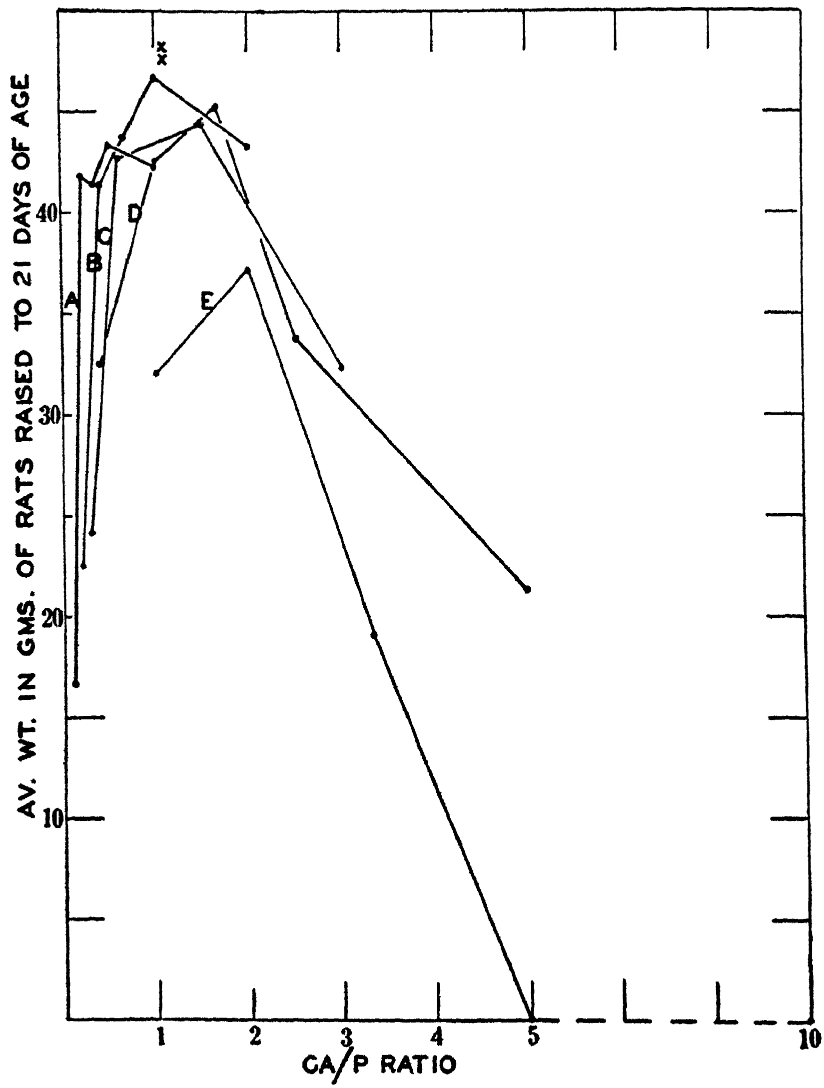

Fig. 2 Average weights of rats raised to 21 days of age by mothers on various experimental diets, against the Ca/P ratio of the diet. Calcium levels are indicated by letters: Curve A is the 0.245 per cent level; B, 0.490 per cent; C, 0.735 per cent; D, 1.225 per cent; and E, 2.45 per cent. The weight of the young from the control mothers is indicated by 'X.'

An examination of the curves warrants the following conclusions:

1. Variable success was obtained even at a Ca/P ratio of 1.0. Performance at this ratio varied from 53 per cent to 96 per cent, and seemed dependent on the *level* of intake of the two elements.

2. By inspection, an average curve, composed of curves A, B, C and D would show a broad maximum extending from a Ca/P ratio of about 0.5 to 1.5 with a peak at, or very near to, 1.0. This would indicate that at all levels of calcium intake, save the maximum employed, a Ca/P ratio of 1.0 or slightly greater is to be preferred.

3. Examined separately, curves A, B, C and D show that *no one Ca/P ratio* can be defined as optimal for gestation and lactation, for the optimum ratio depends on the *level* of calcium intake. At the low calcium level (curve A) best performance was at a Ca/P ratio of 0.33. As the level increased progressively (curves B, C and D), the optimum ratio likewise increased in the order, 1.0, 1.5 and 1.66, respectively.

Weight of young at 21 days. We have separately studied each of the component criteria that have gone into the final success grade. Disregarding any arbitrary grading system we have plotted in figure 2 the average weight of rats raised by each experimental group, against the Ca/P ratio of the diet. All conclusions, detailed above, are confirmed.

The validity of conclusion 3 is emphasized in the following table showing the average maximum weights at each level of calcium intake:

TABLE 3

Relation of calcium level and ratio to average maximum weight of young

DIET NO.	Ca LEVEL PER CENT	Ca/P RATIO	AVERAGE MAXIMUM WEIGHT OF RATS	NUMBER OF RATS
2	0.245	0.50	43.32	156
7	0.490	1.00	46.66	188
12	0.735	1.50	44.43	179
18	1.225	1.66	45.35	159
24	2.450	2.00	37.33	110

The actual differences in average weight between the maximum value and that adjacent to it on any curve is frequently small. We have determined the statistical significance of such differences, and the results are compiled in table 4. Only one significance ratio is less than 3.0 (2.8) so that the differences, though small, are significant, and the conclusion based on them, valid.

TABLE 4

The significance of the maximum weights of the five curves of figure 2

CURVE	CALCIUM LEVEL, PER CENT	(1) MAXIMUM		(2) RIGHT OF MAXIMUM		(3) LEFT OF MAXIMUM		SIGNIFICANCE RATIO [2]	
		Number of observations	P.E.[1] of average weight	Number of observations	P.E.[1] of average weight	Number of observations	P.E.[1] of average weight	(1)–(2)	(1)–(3)
A	0.245	156	0.34	190	0.21	192	0.27	2.8	4.4
B	0.490	188	0.30	206	0.21	112	0.33	9.3	6.7
C	0.735	179	0.34	163	0.26	182	0.25	28.3	4.0
D	1.225	159	0.38	188	0.23	141	0.25	26.3	6.4
E	2.450	110	0.38	57	0.37	82	0.54	34.1	5.7

[1] Probable error. Formula $P.E._m = 0.8453 \times$ the average deviation, divided by the square root of the number of observations.

[2] Calculated by dividing the square root of the sum of the squares of the probable error of the two means into the difference between the means. When the significance ratio is 3.0 or greater the difference is considered statistically significant.

Body ash of young at 21 days. In figure 3 we have plotted the grades obtained by formula (2) against the Ca/P ratio of the diet.

Disregarding the exceptionally high values at very low Ca/P ratios it is evident that the highest grades at all levels of calcium intake were obtained at a ratio of 1.0. This is somewhat surprising in view of the former finding that the optimum ratio for weight was dependent on the level of calcium intake; but it is not inconsistent with this finding. Obviously, maximum weight may not be paralleled by maximum ash.

Thinking in terms of 'grade' magnifies the numerical differences of percentage ash.[8] Thus, curve A shows grades of

[8] To calculate percentage body ash from the grade, multiply the latter by 0.00775, and add 2.000.

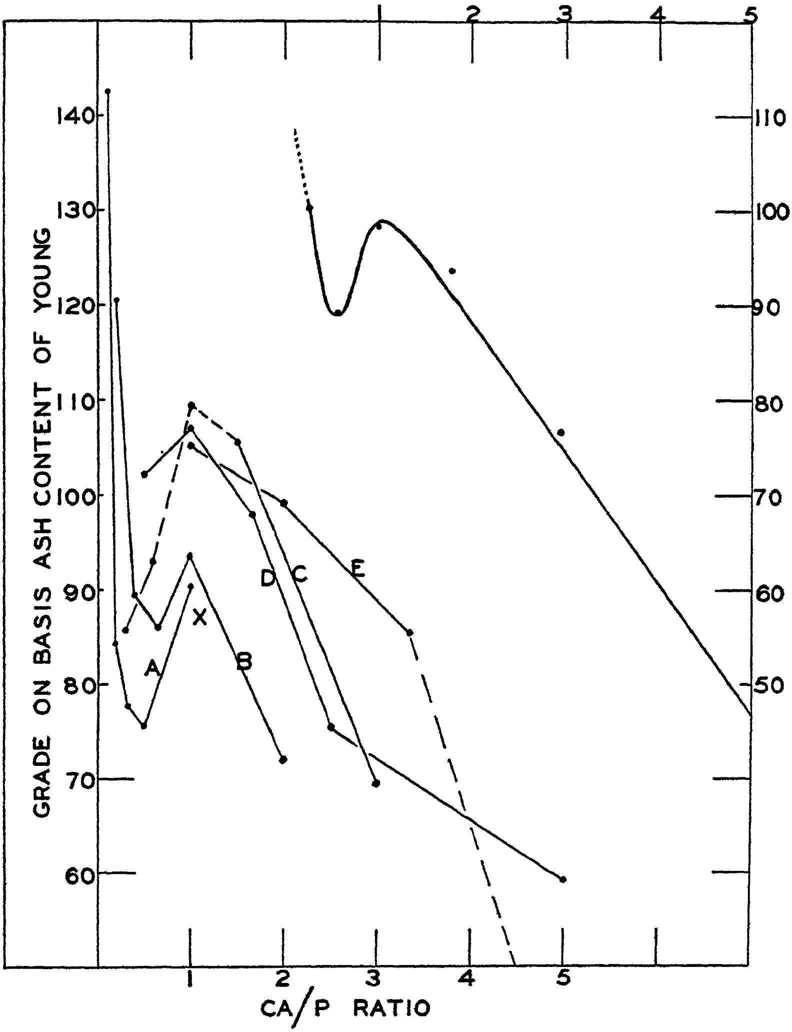

Fig. 3 Total ash contents of 21-day-old young raised by mothers on various experimental diets, against the Ca/P ratio of the diet (formula 2). Calcium levels are indicated by letters: Curve A, 0.245 per cent level; B, 0.490 per cent; C, 0.735 per cent; D, 1.225 per cent; and E, 2.45 per cent. Diet no. 13 was included. The ash content of young from the controls is indicated by 'X.' By moving the coordinates an average curve was constructed in the upper right of the figure.

90.5 and 75.5 at Ca/P ratios of 1.0 and 0.5, respectively. These grades correspond to ash percentages of 2.70 and 2.59—a difference of only 0.11 per cent ash. This difference is statistically significant, as indicated in the following table.

TABLE 5

The significance of maximum ash percentages of the five curves of figure 3

CURVE	CALCIUM LEVEL, PER CENT	(1) MAXIMUM		(2) RIGHT OF MAXIMUM		(3) LEFT OF MAXIMUM		SIGNIFICANCE RATIO	
		Number of observations	Probable error	Number of observations	Probable error	Number of observations	Probable error	(1)–(2)	(1)–(3)
A	0.245	35	2.51	..	...	33	2.40	..	4.2
B	0.490	38	2.17	40	2.03	28	3.81	7.3	1.8
C	0.735	17	3.53	35	3.28	37	2.12	0.8	4.0
D	1.225	29	3.04	35	3.33	24	3.34	2.0	1.1
E	2.450	19	3.37	21	4.35	...	..	1.1	..

The significance ratios in this table indicate that not all differences are really significant. This is partly accounted for by the very large probable error, which, in turn, was due to averaging litters from successive cycles. Usually, initial high ash grades, dropped to very low grades toward the end of the experiment. This is an expression of the strain on mineral resources caused by very rapid reproduction.

At extremely low Ca/P ratios, anomalously high grades were recorded. If diets 5 and 26 are compared (table 2) it will be seen that although very small young (16.6 and 18.9 gm., respectively) were raised to 21 days on both diets, that there was a great difference in the ash of these young. Without definite data on their water content we interpret this to mean that the young raised on diets very high in phosphorus were dehydrated, and that the tissue mineral elements were sufficiently concentrated to account for the high ash content. This thought is further confirmed by our studies on the bone ash of these young, which are reported separately (Cox and Imboden, '36). In spite of a high total ash content, their per cent bone ash was low. On such diets, therefore, total ash content is no indication of bone calcification. Diet no. 26,

however, with a Ca/P ratio even more severe than that of diet no. 5 produced rats equally small, but with normal ash content and normal bone ash (Cox and Imboden, '36). We are inclined to attribute the small size of these young to the inability of the mother to lactate sufficient quantities of milk and calcium on an extremely low calcium diet.

Excessively high ash grades were found only when the Ca/P ratio was more severe than 0.3, and the phosphorus level 2.45 per cent. Thus, the rats on diet 20 (2.45 per cent P, Ca/P ratio 0.5) did not show a greatly lowered bone ash, and did not have, presumably, a high phosphorus rickets (compare their percentage bone ash, Cox and Imboden, '36).

Change in weight of mothers. When formula (3) was applied to the changes in weight of the mother rats, and the results (table 2) graphed, curves similar to those of figures 1 and 2 were obtained. They are not presented here because of lack of space. It was evident that mother rats raising large young gained well in weight, while those that raised small young lost weight.

Because of the following itemized findings, we feel that the mineral content of diet no. 7 (Ca/P ratio 1.0, calcium level, 0.490 per cent) is optimal for gestation and lactation in rats:

1. The success rating was higher than that of any other group (fig. 1).
2. The average weight of young raised was exceeded only by the controls (fig. 2).
3. The grade on body ash was very close to 100, and exceeded that of the controls (fig. 3).
4. The mother rats gained, during the experiment, more than the controls. Diet 7 was one of five groups exceeding the controls in this respect (table 2).

The effect of raising dietary calcium. When diets of low, but constant, phosphorus content are compared, the effect of raising the calcium level is apparent (fig. 4). Presented graphically for successive cycles, the success rating seems regulated by the calcium level. At a very low level (curves

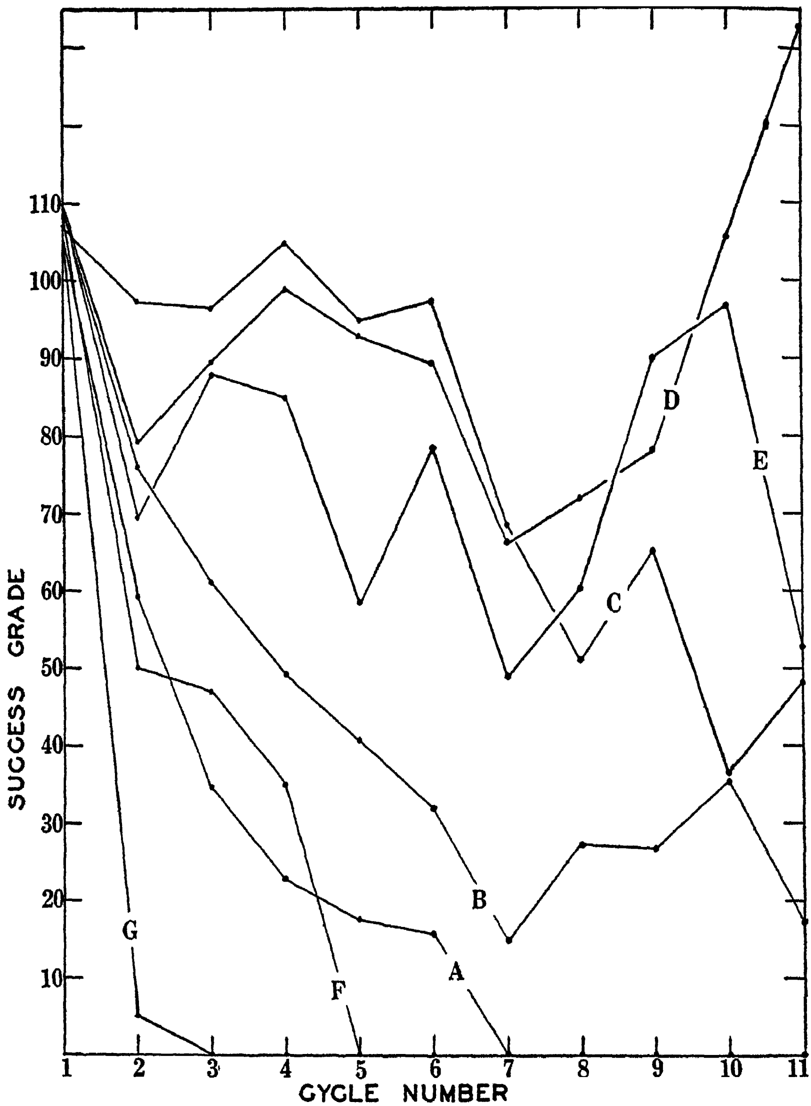

Fig. 4 The 'success' grade of various experimental groups at a constant phosphorus level (0.245 per cent), but various levels of calcium, plotted for successive cycles. Calcium level of curve A is 0.017 per cent; B, 0.122 per cent; C, 0.245 per cent; D, 0.490 per cent; E, 0.735 per cent; F, 1.225 per cent, and G, 2.45 per cent.

A and B) progressive decline in reproductive success was the prominent feature. At higher levels (curves C and D) excellent performance was obtained, and even at a Ca/P ratio of 3.0 (curve E) no great deleterious effect was noted. However, at still higher calcium levels (curves F and G) rachitic diets were indicated by progressively poorer performance.

The point we wish to emphasize is that although success on a given diet may be measured as relatively good for a single cycle, the true adequacy or inadequacy of the dietary intake can only be determined subsequently. An examination of curves B and D at cycle no. 2 (first experimental cycle) and a comparison of these first grades with later ones, indicate this fact. Similarly, curve C shows excellent performance (equalling that of the controls) for six cycles, and only in the last four cycles does the mineral inadequacy of the diet become apparent. Comparison with curve D indicates how performance can be consistently sustained even in the later cycles.

The success ratings of these diets are presented in table 6.

TABLE 6

Effect of increasing calcium intake

CURVE LETTERED	PER CENT Ca	PER CENT P	DIET NO.	AVERAGE SUCCESS RATING
A	0.017	0.245	26	15.0
B	0.122	0.245	27	38.1
C	0.245	0.245	1	76.0
D	0.490	0.245	6	90.4
E	0.735	0.245	11	72.8
F	1.225	0.245	16	13.2
G	2.450	0.245	21	0.5

Excess calcium vs. excess phosphorus. In the plan of our experiment there were equal numbers of groups having Ca/P and P/Ca ratios greater than 1.0. We therefore had an opportunity to determine which element is better tolerated in excess, calcium or phosphorus. Disregarding the mineral level, we have plotted in figure 5 the average weights of young raised against the Ca/P or P/Ca ratio of the diet. The position of the diet, on either side of the center ratio (1.0)

depended upon which element was in excess. Diets with identical ratios were averaged so that the relatively low position of the point at Ca/P ratio 1.0 is due to the low average weight from diet no. 25.

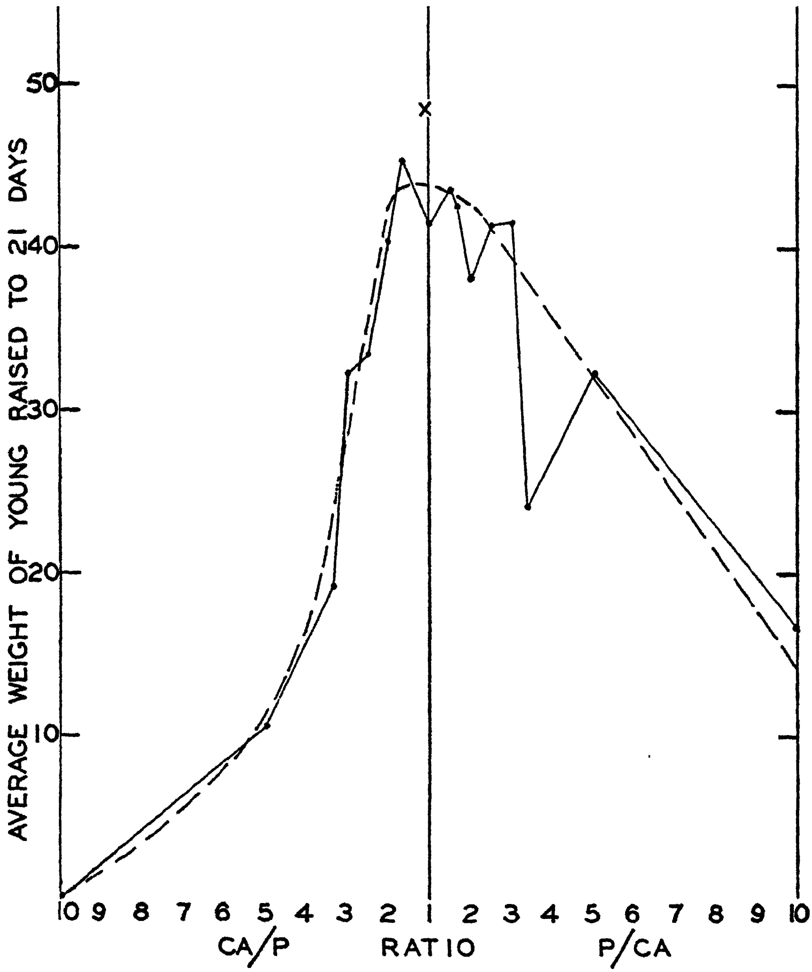

Fig. 5 The average weight of young raised to 21 days by mothers on different experimental diets, plotted against the Ca/P or P/Ca ratio of the diet. The position of the diet on either side of the central line (ratio 1.0) was determined by which element (P or Ca) was in excess. An equal number of experimental diets are recorded on either side of the central line. Groups with identical ratios were averaged. The grade of the controls is indicated by 'X.'

It is immediately evident that on the high calcium side of 1.0, the curve is very steep, and ascends rapidly, while on the high phosphorus side, the curve starts higher (indicating young of greater weight) and the ascent is much more gradual.

Averaging the weights for all diets on the high calcium side (omitting all with Ca/P ratios of 1.0), we obtained a net average weight of 27.7 gm.; while a similar average on the high phosphorus side gave an average of 35.0 gm. The conclusion is obvious; i.e., that phosphorus in excess is better tolerated by rats, than is calcium.

Bone ash of mothers. At the end of the experiment all mothers were killed, and the bone ash of the right femur determined in the usual way. The percentage bone ash (table 2) gave but little indication of the true condition of the bone. In general, it may be said, that diets high in phosphorus (nos. 5, 10, 15, etc.) gave bones extremely thickened, and soft. The bones were easily broken on removal, and frequently had multiple fractures which had healed with large, deforming lumps of bony (but not hard) material. We would term the bones fibrotic, were it not for the fact that the mineral matter was not reduced. Such bones were likewise found with diet no. 25, even though the calcium-phosphorus intake was balanced. Rachitic diets did not give bones especially low in ash, and the greatest reduction was observed in those diets most deficient in calcium, nos. 26 and 27.

Tumors. It has been shown, clinically (Babcock, '35), that lactation does not increase the incidence of mammary tumors. Our experimental animals, however, were afflicted to a surprising degree with such growths. As this is in contradistinction to the incidence in our stock colony, where prolonged rest periods between cycles are allowed, we were inclined to think that the large incidence was related to the rapid sequence of lactation required in this work. More recent observations, however, have made us believe that age is possibly an important factor. McCay, Crowell and Maynard ('35) have recently reported a tumor incidence of 16 per cent in stock animals. An attempt was made to remove surgically all tumors as soon as they were noted. Dr. W. C. Caldwell

examined histologically three typical examples (diet nos. 3, 9 and 12) and found them benign fibro-adenomas of the breast. Histologically, he noted considerable calcium deposits.

In table 2 the number of rats in each group showing tumors is recorded. Obviously there was no dietary distribution.

Calculi. Recent experimental work (Higgins, '33) has confirmed earlier observations (Osborne and Mendel, '17) that prolonged deficiency of vitamin A may cause bladder and kidney stones. Obviously, other factors are also involved (Keyser, '35). Our rats received adequate amounts of vitamin A (as carotene) but on certain diets, high in calcium, stones were observed; viz., diet 11, one rat; diet 18, one rat; diet 22, one rat; diet 23, two rats. On the latter diet (see plate 1, actual size), rat no. 115 had five bladder stones weighing 2.955 gm. (dried), and rat no. 114 had the entire renal calyx literally packed with calcium deposits. Rat no. 85 (diet 18) had three bladder stones, and much 'sand.' Plate 1 shows the relatively enormous size of these deposits. The largest stone measured $1.47 \times 1.02 \times 0.80$ cm. Analysis showed the stones to consist almost entirely of calcium salts with only traces of phosphate (table 7).

TABLE 7

Analysis of renal calculi

STONE FROM RAT NUMBER	PER CENT ASH	IN CALCULUS			IN ASH		
		Per cent Ca	Per cent P	Per cent Mg	Per cent Ca	Per cent P	Per cent Mg
85	52.54	34.69	0.78	1.22	66.03	1.48	2.32
115	52.29	34.60	0.86	1.39	66.17	1.65	2.65

Food and water intake. For six experimental cycles record was made of food and distilled water intake. The average figures are given in table 2. Caloric consumption during gestation was, to some extent, dependent on the mineral level and ratio of the diet. A low calcium level (diet nos. 26 and 27) did not result in increased food consumption. High calcium or phosphorus levels at unfavorable ratios (diet nos. 5, 10, 15, 21 and 22) resulted in decreased food consumption. During lactation the caloric consumption increased to ap-

proximately the same extent on all diets (calculated per pup) save those most unbalanced in mineral elements. The increase due to lactation was greatest in the stock controls. Water intake was roughly equivalent in all groups, save those high in phosphorus. On such diets (4, 5, 10, 15 and 20) abnormal quantities were ingested. On the other hand, diets high in calcium did not cause such increases. In partial explanation, phosphorus is largely excreted in the urine; calcium is unabsorbed, or normally excreted through the large bowel, and therefore does not require additional fluid for its elimination.

DISCUSSION

Our results in this investigation have led us to the view that the optimum Ca/P ratio for gestation and lactation in rats is dependent upon the calcium level, the optimum ratio increasing as the calcium increases. This finding was based upon a study of our 'success ratings,' and the average weight of young raised by the different groups of mothers. On the other hand, a consideration of the body ash of the weaned young indicated that at all calcium levels a Ca/P ratio of 1.0 gave the greatest percentage ash, although all differences could not be regarded as statistically significant.

When our data was rearranged and examined at constant phosphorus levels, clear cut results were not obtained. We employed two criteria, success ratings and weights of young. The findings are presented in table 8.

When we examined the statistical significance of the average weights at a given phosphorus level, it was found that, at some levels, the average weight at the optimum ratio was not significantly different from the average weight at some other ratio. This is indicated in the last column of table 8.

We feel that the results are not sufficiently straight-forward to permit generalization. For example, it might be maintained that at all levels of phosphorus a P/Ca ratio of 1.0 was optimal; and with perhaps equal certitude, that the optimal P/Ca ratio increased with the phosphorus level until phosphorus constituted 0.735 per cent of the diet.

During the course of this work we were surprised that many of our rats, receiving a synthetic diet, performed as well as, or better than, the controls. This is in contradistinction to other reports on synthetic diets (Hartwell, '33). We are inclined to credit such results to an adequacy of the B vitamins. These were included at a high level because of the increased requirement during lactation (Sure, '27).

The decline in ash content of the young (with successive litters) does not appear when the average ash contents are reported. Such a decline was true in the control litters as well as in the experimental groups, although we had hoped that the ash percentage of the former would remain relatively constant throughout successive cycles. Sherman and MacLeod ('25) have stated that the calcium content of virgin females is higher than that of males, but that after they had raised young, their calcium content resembled that of males. The decline in percentage ash of the young noted in this work was accompanied by an increase in the average weight, so that the actual weight of ash in their bodies increased (fig. 6). The fact that young from older mothers are larger, has been noted before (Abt, '23). Graphic record of the behavior of our stock control groups is given in figure 7.

TABLE 8

Relation of phosphorus level and optimum P/Ca ratio (compare table 3)

PHOSPHORUS LEVEL PER CENT OF DIET	OPTIMUM P/Ca RATIO		
	Basis: Success rating	Basis: Average weight young	Possible optimum ratio: see text
0.245	0.5	0.5	1.0
0.490	1.0	1.0	...
0.735	3.0	0.6	...[1]
1.225	1.66	1.66	1.0
2.450	1.0	1.0	...

[1] Diet 13, ratio 1.0, was not included. See page 153.

Because the lactating animal is able, physiologically, to make adjustments between dietary intakes of calcium and phosphorus, and that supplied to the nursing young in the milk, the wide differences that have been noted in the growth of weaned young when placed on such severe ratios as we have employed, were not observed in this work. At a Ca/P

ratio of 0.25 (compare our diet nos. 3 and 4, fig. 2) Bethke, Kick and Wilder ('32) observed their poorest growth, irrespective of the presence of vitamin D. The weight of young weaned on such diets, as reported here, was exceptionally good. Similarly, our results with a Ca/P ratio of 3.0 (diet no. 11) might be interpreted as relatively better than the growth reported by these authors for a diet with a similar ratio at a low level. To assure the well-being of her young, the lactating mother may be expected to make the maximum

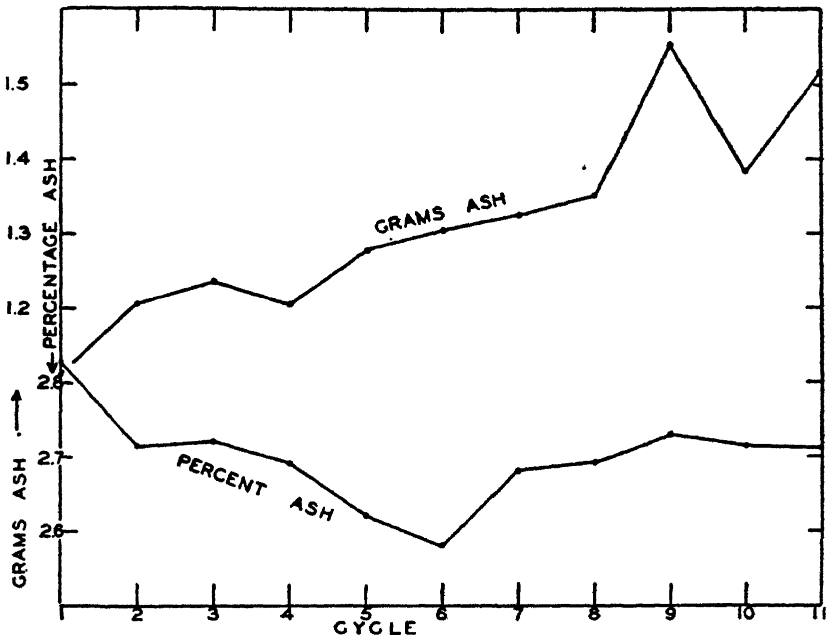

Fig. 6 The per cent ash and grams of ash in the bodies of 21-day-old young raised by the control mothers, plotted for successive cycles.

possible adjustment in her milk. This is indicated by the data of Huffman, Robinson and Winter ('30). We have calculated from their reported data the change in the calcium and phosphorus content of the milk of lactating cows on different levels of mineral intake at different levels of milk production (table 9).

In spite of doubling the mineral intake, the percentage of calcium and phosphorus remained constant. An interesting

TABLE 9

Calculated from the data of Huffman, Robinson and Winter ('30)

MILK PRODUCTION	AVERAGE MILK DAILY	INTAKE		COMPOSITION OF MILK	
		Ca	P	Per cent Ca	Per cent P
	kg.	*gm.*	*gm.*		
Low	8.2	32.3	32.6	0.115	0.090
	11.2	77.9	61.3	0.111	0.090
High	27.3	49.7	57.0	0.109	0.088
	29.5	131.9	104.7	0.116	0.090

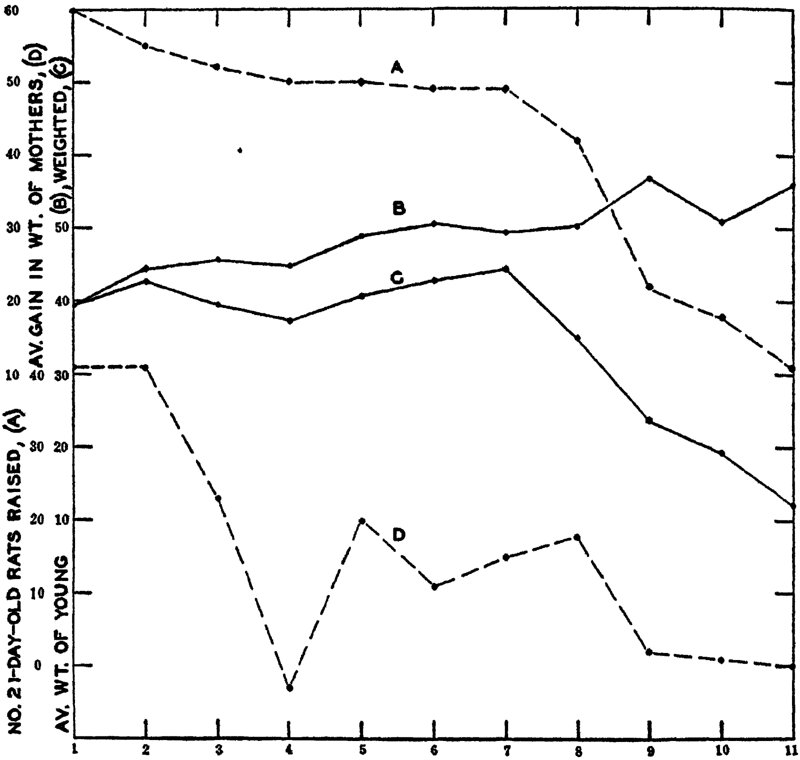

Fig. 7 The behavior of control mothers for successive reproductive cycles. Curve A shows the total number of young raised by these mothers; Curve B, the average weight of these young; Curve C, the corrected weight (corrected for the number that should have been raised) and, Curve D, the average weight fluctuations of the mothers themselves.

problem for further investigation is the determination of the effect of different Ca/P ratios on the Ca/P ratio of the milk.

We have thought it worth while to calculate from the calcium intake of our experimental rats, what would be the equivalent intake by human mothers. We have made such a comparison on the basis of weight, surface area and caloric intake.[9]

[9] Comparison of weight, surface area and caloric intake of woman and rat during reproduction.

	A *Rat*	*B* *Woman*	$\frac{B}{A}$
Weight			
Average non-pregnant	300 gm.	58 kg.	193
Increase due to pregnancy	87 gm.[1]	9.1 kg.[2]	
Weight at term	387 gm.	67.1 kg.	173
Surface area[3]			
Height (in cm.)	...	160 cm.	
Calculated surface area	408 sq.cm.	1.60 sq.m.	39
Calculated surface area at term	485 sq.cm.	1.70 sq.m.	35
Caloric intake			
Basal metabolism cal./sq.m.	766[4]	888[5]	
Basal requirement, in calories	31.3	1421	
Normal caloric intake	57.5[6]	2730[7]	
Per cent increase over basal requirements	183.7	192.1	
Per cent heat increment in last 0.4 of pregnancy	12.5[8]	12.5[9]	
Caloric increase in last 0.4 per cent of pregnancy	3.9	178	
Distributed over whole pregnancy	1.6	71	
Calculated caloric intake during pregnancy	60.4[10]	2865	45
Determined caloric intake[11]	63.9	....	

[1] Experimentally determined on forty-nine stock animals. Increase in weight was 3.22 per cent per pup. Average young, nine. Average gain in weight during gestation 29 per cent.

[2] Many authorities cite 20 pounds as the desired gain in weight.

[3] Formula, for rats, $S = 9.1 \times W^{0.67}$; for women, $S = H^{0.725} \times W^{0.425} \times 71.84$.

[4] Benedict and McLeod, J. Nutrition, vol. 1, p. 373 (1929). Average value for 15-month-old rats in winter and summer at 28°C.

[5] Du Bois, E. F., Basal metabolism in health and disease.

[6] Experimentally determined on 300 gm. resting mother rats.

[7] Average of caloric intakes given by Lusk for housemaids and laundresses at 8-hour hard and medium work, with an additional 10 per cent for fecal loss. See Shukers, C. F., Macy, I. G., Donelson, E., Nims, B., and Hunscher, H. A., J. Nutrition, vol. 4, p. 399 (1931).

[8] Assuming same as for woman.

[9] Calculated from data of Sandford and Wheeler, J. Biol. Chem., vol. 62, p. 329 (1924).

[10] Example: $(31.3 + 1.6) \times 183.7$.

[11] Average of twenty-seven gestations of rats on diet no. 1.

Table 10, based on the calcium intake of rats on diet no. 1, shows that on a weight basis, human mothers would ingest 6.75 gm. calcium daily to equal the intake of such rats during gestation; on a surface area basis, 1.37 gm., and on a caloric basis, 1.75 gm.

Comparisons on the basis of surface area are more dependable than those on weight. If we can tentatively accept 1.37 gm. as the human calcium equivalent of diet no. 1 (calcium level 0.245 per cent, success grade 76.0), then diet no. 7 (calcium level 0.490 per cent, success grade 95.8) is approximately equivalent to 2.74 gm., and diet no. 27 (calcium level,

TABLE 10

Theoretical calcium intake of woman compared to rat on weight, area and caloric basis

COMPARED ON BASIS	INTAKE Ca PER RAT PER DAY (DIET NO. 1)	FACTOR B/A FOOTNOTE 9	THEORETICAL DAILY CALCIUM INTAKE OF WOMAN	
			During gestation	During lactation
	gm.		*gm.*	*gm.*
Weight	0.039	173	6.75	
	0.044	193		8.49
Area	0.039	35	1.37	
	0.044	39		1.72
Caloric intake	0.039	45	1.75	....

0.122 per cent, success grade 38.1), to 0.63 gm. If it were possible to place women under experimental conditions similar to those described here and assure the three mineral intakes mentioned, one might expect curves similar to B, C and D of figure 4. The average calcium intake of American women does not greatly exceed 0.63 gm. (Sherman, '32; Schmidt and Greenberg, '35). On the basis of their balance studies, Toverud and Toverud recommend 1.5 to 2.0 gm. daily, while most authorities feel 1.0 to 1.5 gm. is adequate during gestation. At this low calcium level (0.245 per cent, equivalent to 1.37 gm.), a Ca/P ratio of less than 1.0 would probably be optimal (compare figs. 1 and 2). A ratio less than one is recommended by Sherman ('32) for normal adult maintenance, and Toverud ('33) for gestation. It is not clear why

a ratio less than 1.0 is preferable at low calcium levels. Possibilities that suggest themselves are: 1) the requirement of phosphorus for tissue growth is relatively greater during this period and is in excess of the minimal calcium level at which skeletal growth can proceed, or 2) at ratios greater than 1.0 there is more calcified body tissue.

SUMMARY

1. One hundred and forty-five female rats (who gave birth to 8431 pups, and raised 4391 to 21 days of age) have been studied for ten consecutive reproductive cycles while receiving a diet of purified foodstuffs containing a constant quantity, but varying levels and ratios, of calcium and phosphorus.

2. Both level and ratio of mineral elements exerted an effect on the success of the mothers in producing and rearing young.

3. A Ca/P ratio of 1.0, at a calcium level of 0.490 per cent, was adjudged (on the basis of weight of young at 21 days, ash content of the young, and change in weight of the mothers) the ideal mineral level and ratio for successful gestation and lactation in rats.

4. By employing the average weights of 21-day-old young it was shown that the optimal ratio depends upon, and is proportional to, the calcium level. If the calcium intake is not known exactly, a Ca/P ratio of 1.0 will approximate the optimal.

5. At excessive mineral levels (2.45 per cent) poor performance was obtained, irrespective of ratio.

6. The largest ash contents of the 21-day-old young were uniformly obtained when the mother rat received a diet with Ca/P ratio of 1.0.

7. At constant phosphorus intake (0.245 per cent), increasing the calcium content of the mothers' diet from 0.017 to 0.490 per cent gave better reproductive success; further calcium increases made for rachitic diets and failure.

8. Phosphorus (PO_4) in excess was better tolerated than calcium in excess.

9. The relationship of these findings to human nutrition has been discussed.

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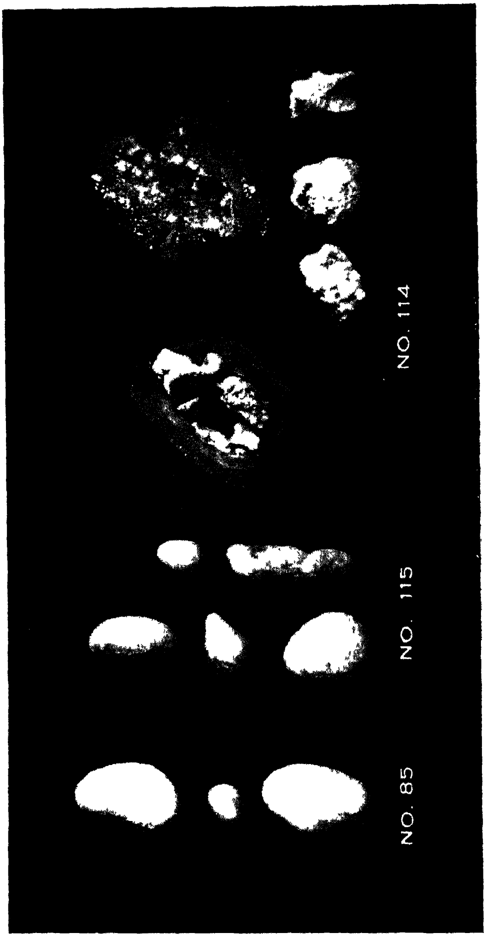

Photograph showing the actual size of urinary and renal calculi obtained from three rats. Rat no. 85 was on diet no. 17. Rats nos. 114 and 115 were on diet no. 23.

THE MINERAL COMPOSITION OF YOUNG RATS

WARREN M. COX, JR. AND MIRIAM IMBODEN
Research Laboratory, Mead Johnson & Co., Evansville, Indiana

FIVE FIGURES

(Received for publication September 18, 1935)

In the preceding paper (Cox and Imboden, '36) we drew attention to the effect of different levels and ratios of calcium and phosphorus in determining reproductive success.[1] During this work we had followed more than 8000 young rats (inbred Wisconsin strain) and had compiled considerable data on the inorganic composition of the young, and the reproductive behavior of mother rats. In the following sections we present these additional data.

Effect of dietary minerals on the composition of the new-born rat. One hundred and forty-five mother rats gave birth to 8431 pups during the course of our work. The young were weighed on the day of their birth (usually they had suckled—as indicated by a white patch across the belly), and 522 of them analyzed for calcium and phosphorus, after ashing the entire rat. In table 1 the composition of first litter pups from mothers which received stock food, is compared with that of pups from mothers on twenty-four experimental diets, containing different mineral levels.

In spite of the fact that there was a great difference in the mineral content of these experimental diets the composition of the pups was within the range observed in analyses of stock rats, and the averages were practically identical (table 1). Certainly it can be said that a change in dietary minerals

[1] The composition of experimental diets referred to in this work is given in the previous paper.

is not immediately reflected by a change in the composition of fetal tissue, and, conversely, that the maternal organism is able to regulate the mineral elements supplied to the fetus.

However, not all mineral levels can be so compensated. Diets excessively high in calcium and unbalanced as regards

TABLE 1

Composition of rat pups

DIET	NUMBER OF ANIMALS	AVERAGE WEIGHT, GRAMS	NUMBER OF DETERMINATIONS	ASH		CALCIUM		PHOPHORUS		OBSERVER
				Per cent	A.D.[1]	Per cent	A.D.[1]	Per cent	A.D.[1]	
Stock first litter	239	5.37	50	1.754	0.056	0.269	0.017	0.280	0.028	....
Experimental diets, various second litter	263	5.12	53	1.730	0.060	0.269	0.021	0.294	0.011	....
Diets 21 to 23 second litter	20	4.89	6	1.512	0.077	0.195	0.037	0.233	0.016	....
Reports of other investigators										
Stock	166	....	..	....	....	0.260	....	0.295	....	Goss and Schmidt ('30)
Stock	15	4.7	..	....	....	0.250	....	....	....	Sherman and McLeod ('25)
Stock	21	4.3	..	....	....	....	....	0.34	....	Sherman and Quinn ('26)
Various Ca levels	37	4.4	..	....	....	0.28	....	....	....	Sherman and Booher ('31)
Stock	...	....	..	1.576	....	0.271	....	0.267	....	Toverud ('23–'24)

[1] Average deviation.

phosphorus, nos. 21 to 23, did adversely affect the composition of the newly born, as indicated in table 1. Other diets, notably diet 16, Ca/P ratio, 5.0, gave a lowered ash content of the young after several successive reproductive cycles. These young are being examined histologically[2] and it may be said,

[2] By Dr. E. A. Park, Harriet Lane Home, Johns Hopkins University.

in advance of publication, that they show evidence of experimental congenital rickets.

Booher and Hansmann ('31–'32) have noted the constancy of the percentage bone ash in newly born infants, in spite of what was judged to be large differences in maternal intakes of calcium and phosphorus. Similarly, Macomber ('27) has shown that diets low in calcium do not cause a reduction of the calcium content of the rat at birth; he attributes this lack of effect to the relatively low calcium content of the rat fetus. The cartilagenous nature of the rat at birth as compared to the human infant at birth (Booher and Hansmann, '31–'32; White House Conference, '32) is illustrated in table 2.

TABLE 2

The inorganic composition of the rat, and human infant at birth

	WEIGHT	PER CENT ASH	TOTAL WEIGHT		PER KILOGRAM BODY WEIGHT		PER CENT ASH		Ca : P RATIO IN ASH
			Calcium	Phosphorus	Calcium	Phosphorus	Calcium	Phosphorus	
	gm.		gm.	gm.	gm.	gm.			
Rat pup	5.37	1.754	0.0144	0.0150	2.68	2.79	15.3	16.0	0.96
Human fetus[1]	3000	3.33	24.21	13.32	8.07	4.44	24.2	13.3	1.82
21-day-old rat[2]	44.7	2.738	0.313	0.236	7.01	5.27	25.7	19.3	1.33

[1] Full term.

[2] Average of 420, 21-day-old stock rats.

Such a comparison indicates that gestation per se causes little strain on the mineral stores of mother rats, in contradistinction to the considerable strain of gestation on human mothers. Thus, the lack of mineral demand during gestation in rats makes the balance experiments of Goss and Schmidt ('30), in which they showed consistently positive calcium balances in rats during pregnancy, more readily understandable. The composition of the rat, in terms of ash, calcium and phosphorus, approaches that of the infant at birth, only after the former has suckled some 21 days.

Body ash at 21 days of age

To establish standards for our rat colony, and for purposes of comparison we have determined the total body ash of 420 stock rats 21 days of age, 570 rats less than this age and twenty-three rats 43 days old. In figure 1 we have plotted the weight of rat against the weight of the ash[3] (the only 21-day-old rats used in this curve were those known to be the first

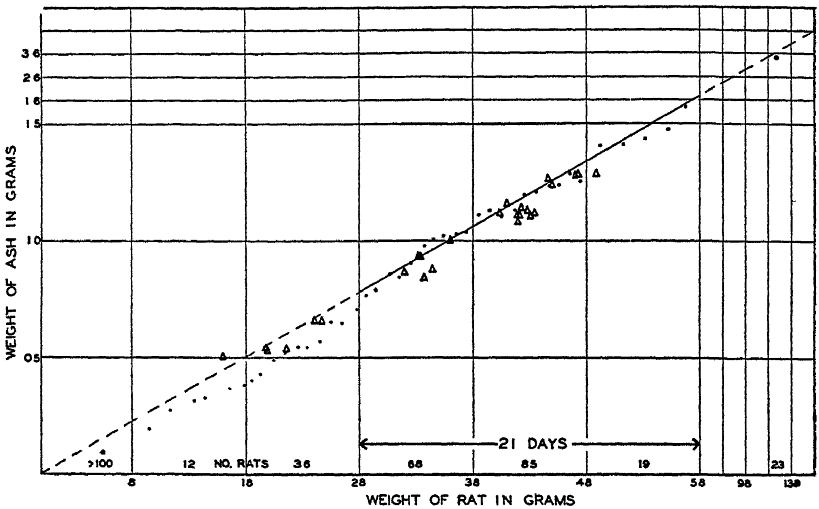

Fig. 1 Weight of rats plotted against the weight of ash. The range of weight of 21-day-old, first litter rats from stock mothers is indicated by an arrow. The small dots indicate averages—averages below 28 gm. indicate rats younger than 21 days. Triangles are averages of 21-day-old rats whose mothers received experimental diets with different amounts of calcium and phosphorus (Cox and Imboden, '36).

young from stock mothers). For 21-day-old rats the average figures gave a straight line, which, when extended, was considerably above the ash content of younger rats, and rats at birth. The triangles are averages of each of the experimental diets (3147 rats were ashed, and the averages used in determining the position of the triangle on the curve) (Cox and

[3] After chloroforming, the rats were weighed on a torsion balance, the gastro-intestinal tract removed, and the carcasses ashed at 550°C. In plotting figure 1, and determining percentages, the gross body weight was used.

Imboden, '36). Most of the values lie fairly close to the control line. It is evident that small rats, 21 days of age, have a larger mineral content than younger rats of the same weight.

The slope of this curve corresponds to an ash percentage of 2.775, and in the evaluation of our experimental rats we employed this value. The actual average of the 420 (various

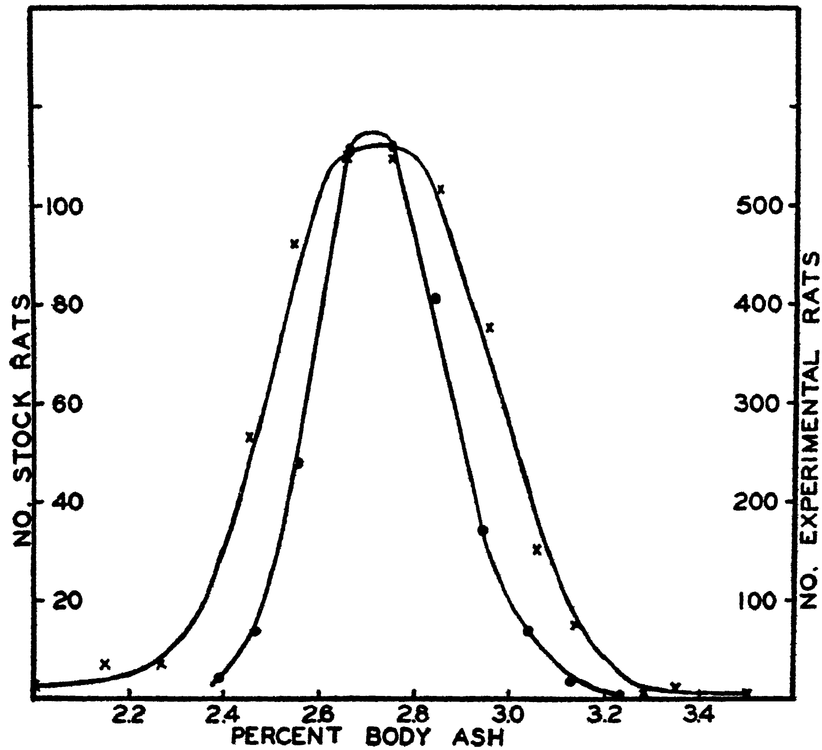

Fig. 2 Distribution, in terms of percentage body ash, of stock rats and experimental animals reported in this work. The curve for experimental animals is indicated by crosses.

litter numbers) 21-day-old rats was 2.738 per cent, and their distribution curve (fig. 2) would indicate that a fair sample had been employed. On the basis of this sample we have determined that an average rat of our stock colony, at 21 days of age, will weigh 44.7 gm. and will contain 1.22 gm. ash (2.738 per cent), 0.701 per cent calcium, and 0.527 per cent

phosphorus. The ash will contain 25.66 per cent calcium and 19.30 per cent phosphorus. There is but very slight variation in the composition of the ash (in calcium $\pm$ 0.44 per cent,[4] in phosphorus $\pm$ 0.37 per cent[4]).

The distribution of the 3147 experimental rats, in terms of percentage ash is given in figure 2. The range of ash content

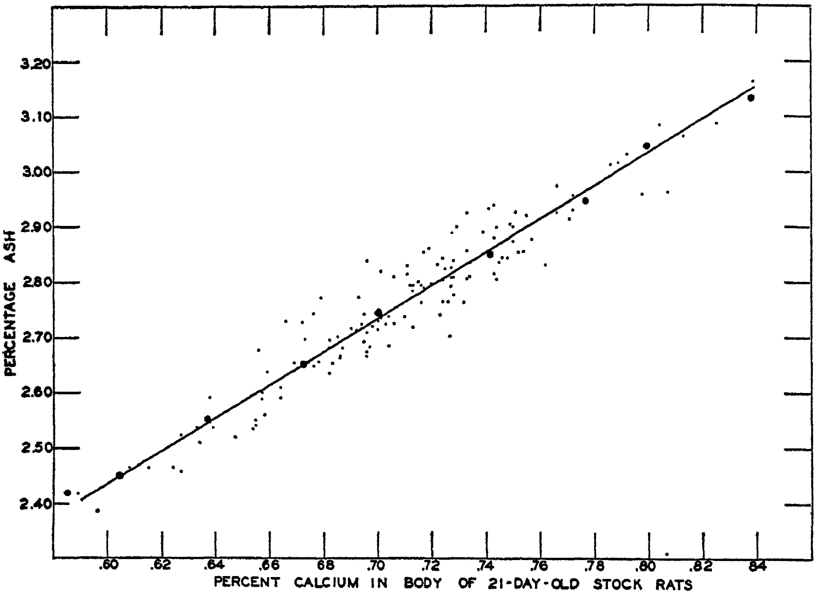

Fig. 3 Per cent calcium in 21-day-old rats against per cent body ash. The smaller dots indicate individual determinations on stock animals. The larger dots are averages of experimental animals (table 3).

is slightly greater than that of the stock rats, but this was to be expected in view of the wide range in mineral content of the diets.

Calcium and phosphorus in 21-day-old rats

Because of the constancy in the composition of the ash it is obvious that the amount of calcium and phosphorus in the bodies of rats is dependent upon the amount of ash. We have illustrated this point in figures 3 and 4, by plotting the

[4] Average deviation.

per cent calcium and phosphorus[5] against the per cent ash. The smaller points indicate single determinations on first litter, 21-day-old stock rats, and the larger points are averages of 544 calcium and phosphorus determinations on ex-

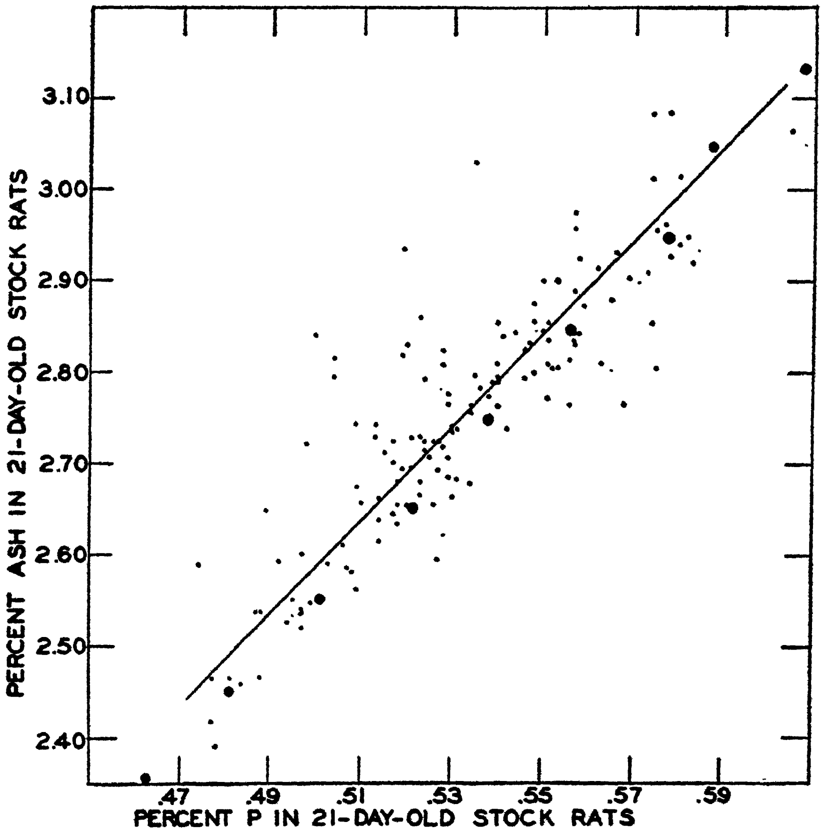

Fig. 4 Per cent phosphorus in 21-day-old rats against per cent body ash. The smaller dots indicate individual determinations on stock animals. The larger dots are averages of experimental animals (table 3).

perimental rats. If the percentage ash is known, the percentage calcium or phosphorus in the body may be read directly from the graph with but little error. The average calcium

[5] Calcium was determined by titration of the oxalate with permanganate—essentially the method of McCrudden ('09–'10), and phosphorus, by the official volumetric method of the A. O. A. C. ('30).

values for the experimental rats agree well with the curve drawn from data on the stock colony, while the values for phosphorus are slightly below those for stock rats.

Table 3 gives in concise form these average values, and the deviations from the average.[6]

TABLE 3

The percentage ash, calcium and phosphorus in 21-day-old rats, whose mothers received diets with different mineral levels (Cox and Imboden, '36)

ASH RANGE	NUMBER OF RATS	PER CENT ASH MEAN	A.D.[1]	CALCIUM		PHOSPHORUS		NUMBER OF DETERMINATIONS
				Per cent in body	A.D.[1]	Per cent in body	A.D.[1]	
1.9–2.1	12	2.008	0.028	0.424	....	0.395	....	2
2.1–2.2	35	2.148	0.022	0.506	0.012	0.422	0.008	8
2.2–2.3	36	2.266	0.019	0.543	0.014	0.445	0.008	7
2.3–2.4	102	2.360	0.025	0.576	0.013	0.463	0.010	28
2.4–2.5	265	2.451	0.028	0.604	0.016	0.481	0.008	48
2.5–2.6	460	2.554	0.023	0.637	0.014	0.502	0.008	81
2.6–2.7	548	2.651	0.024	0.672	0.015	0.521	0.009	95
2.7–2.8	547	2.748	0.022	0.700	0.013	0.538	0.009	91
2.8–2.9	516	2.849	0.023	0.741	0.014	0.556	0.009	81
2.9–3.0	377	2.948	0.024	0.777	0.015	0.578	0.011	58
3.0–3.1	150	3.049	0.023	0.797	0.016	0.587	0.014	28
3.1–3.2	76	3.140	0.021	0.838	0.020	0.608	0.009	12
3.2–3.3	7	3.282	0.013	0.890	....	0.655	....	2
3.3–3.4	11	3.346	0.037	0.907	....	0.649	....	2
3.4–3.5	5	3.499	....	0.939	....	0.672	....	1

[1] Average deviation from the mean. The probable error of the mean may be calculated by Peters' approximation (Munch, '31) $P.E._m = \frac{0.8453 \times A.D.}{\sqrt{n}}$. The significance of the difference between two means may be calculated as given by Smith and Smith ('34).

The relation between body ash and bone ash

It was previously stated (Cox and Imboden, '36) that maternal diets containing different amounts of calcium and phosphorus would cause mother rats to raise young with different percentages of body ash. It has not previously been shown that per cent body ash bears any relation to the commonly accepted measure of calcification; i.e., bone ash. The

[6] We have determined the statistical significance of the differences in per cent ash, calcium and phosphorus. The significance ratios (Smith and Smith, '34) were greatly in excess of 3.0.

effect of Ca : P ratios on calcification has usually been studied by feeding the young, weaned rat, and determining the resultant bone ash. The work in this field has been summarized by Bethke, Steenbock and Nelson ('23–'24). More recently two papers have contributed greatly to our knowledge of this relationship (Bethke et al., '32; Brown et al., '32).

To study the question of the relationship between percentage body ash and bone ash, we routinely, for a time, took

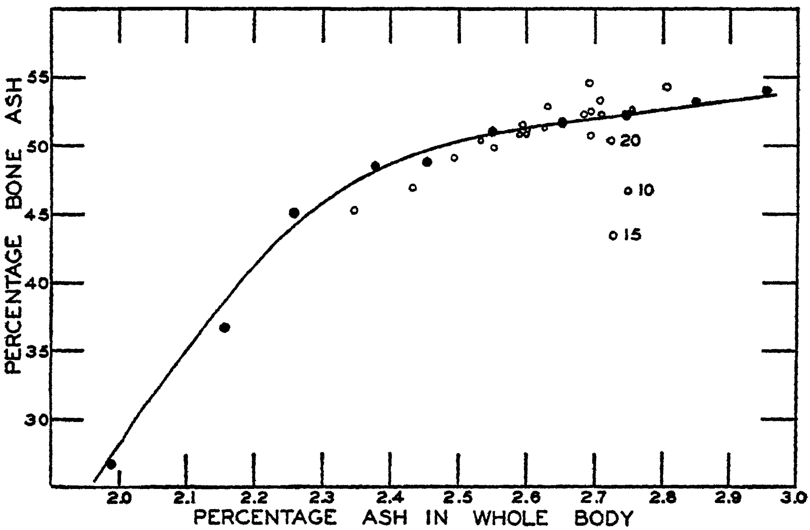

Fig. 5 The per cent body ash of 21-day-old rats plotted against the per cent bone ash of litter mates. Solid black points indicate group averages; circles are averages of rats raised on different experimental diets. The three numbers stated opposite the circles refer to diets nos. 10, 15, etc. (Cox and Imboden, '36).

one rat from each litter raised by mothers on different mineral levels and determined the percentage ash of the dry, alcohol-ether extracted bones (Bethke, Steenbock and Nelson, '23–'24). Both tibiae, humeri and femora were used. The remaining rats in the litter were ashed in an electric muffle after removal of the gastro-intestinal tract. One hundred and fifty-four litters were treated in this way.

When the percentage bone ash was plotted against the percentage body ash of litter mates a smooth curve was obtained. In figure 5 the solid dots indicate group averages;

i.e., as between body ash percentages of 2.5 to 2.6, etc. The differences in bone ash of adjacent points on the more asymptotic part of the curve are not statistically significant, but between alternate points the differences are significant (table 4).

Figure 5 demonstrates that body ash may be used as a measure of bone ash or of the degree of calcification in 21-day-old rats. When the body ash percentage is less than 2.4 per cent (bone ash 48 per cent) there is some degree of inferior calcification.

TABLE 4

Body ash and bone ash values of 21-day-old rats

BODY ASH RANGE	AVERAGE BONE ASH	NUMBER OF DETERMINATIONS	P.E.[1] OF MEAN	REFERENCE NO.	DIFFERENCE BETWEEN	SIGNIFICANCE RATIO (SMITH AND SMITH, '34)
2.2–2.3	45.10	4	0.88	1	...	....
2.3–2.4	48.48	7	0.40	2	...	....
2.4–2.5	48.80	23	0.27	3	3–1	4.51
2.5–2.6	51.00	34	0.21	4	4–2	5.58
2.6–2.7	51.68	36	0.27	5	5–3	7.54
2.7–2.8	52.59	20	0.27	6	6–4	4.64
2.8–2.9	53.22	20	0.38	7	7–5	3.31
2.9–3.0	54.06	8	0.41	8	8–6	2.99

[1] Probable error. Peters' approximation (Munch, '31).

When the determinations were rearranged in accordance with the diet employed (circles), two points were definitely off the curve. These were rats raised on diets nos. 10 and 15 which contained a high level of phosphorus (2.45 per cent) at a Ca/P ratio of 0.2 and 0.3. On such high phosphorus diets the body ash cannot be used as an expression of the degree of calcification. In spite of a normal per cent body ash, the bone ash was sufficiently low to indicate a degree of rickets (high phosphorus rickets). The young were very small (15 to 18 gm.) and we are inclined to attribute the above finding to the concentration of mineral elements in their tissues: an hypothesis not tenable with small rats exhibiting a high calcium rickets.

Calcification of male and female rats

It has been shown that female rats, before reproduction, contain larger amounts of mineral elements than do male rats (Sherman et al., '25, '26, '31; Hammett, '23). We have compiled our figures in which the sex of the rats was recorded, and present the results in table 5. The conclusions of previous investigators are confirmed.

TABLE 5

The mineral content of 21-day-old rats of different sex whose mothers received stock diet

	FIRST LITTER		UNKNOWN LITTER	
	Male	Female	Male	Female
Number of determinations	42	40	31	36
Number of rats	42	49	31	36
Average weight	39.5	39.6	42.9	42.5
Average per cent ash	2.791	2.856	2.783	2.826
Average per cent Ca	0.711	0.740		
Average per cent P	0.537	0.548		
Average per cent Ca in ash	25.46	25.90		
Average per cent P in ash	19.24	19.21		

Successive gestations

We followed ten stock control mothers through eleven reproductive cycles and present in table 6 a record of the effect of successive reproduction and advancing age. In table 7 a similar compilation for the 135 experimental mothers is presented.

Such data on a stock colony (albino) has been previously reported by King ('15, '24), and King and Stotsenburg ('15). A comparison of our results with King's data indicates either the improvement that has taken place in experimental rat colonies, or the wide difference between albino and extracted rats. It is to be especially noted that only 2 per cent of her albino and 21 per cent of her piebald females produced an eleventh litter, whereas 40 per cent of our controls successfully cast an eleventh litter. Gray Norway rats had only an average 3.69 litters during their reproductive life (King and Donaldson, '29). Even on diets of purified foodstuffs (and

various mineral levels) our rats exhibited better average reproduction than King's stock animals.

These tables justify, we feel, the following generalizations: 1) the number of young born is a function of the cycle

TABLE 6

Reproductive record of stock rats for eleven successive cycles

CYCLE NO.	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	AVERAGE NUMBER PER MOTHER	AVERAGE WEIGHT, GRAMS	NUMBER OF YOUNG AVAILABLE TO RAISE	NUMBER OF YOUNG RAISED TO 21 DAYS	PER CENT RAISED	AVERAGE WEIGHT, GRAMS
1	10	98	9.8	5.76	60	60	100	39.19
2	10	93	9.3	5.67	57	55	96.5	44.45
3	10	107	10.7	5.40	60	52	86.7	45.51
4	10	100	10.0	5.66	60	50	83.3	44.72
5	9	83	9.2	5.63	54	50	92.6	48.83
6	9	74	8.2	5.55	52	49	94.2	50.59
7	9	70	7.8	5.55	50	49	98.0	49.44
8	8	71	8.9	5.38	48	42	87.5	50.20
9	7	45	6.4	5.31	37	22	59.5	56.91
10	5	37	7.4	5.42	26	18	69.2	50.97
11	4	23	5.7	5.17	20	11	55.0	56.00

TABLE 7

Reproductive record of experimental rats for eleven successive cycles

CYCLE NO.	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	AVERAGE NUMBER PER MOTHER	AVERAGE WEIGHT, GRAMS	NUMBER OF YOUNG AVAILABLE TO RAISE	NUMBER OF YOUNG RAISED TO 21 DAYS	PER CENT RAISED	AVERAGE WEIGHT, GRAMS
1[1]	135	1388	10.3	5.62	810	800	98.8	40.92
2	128	1253	9.8	5.30	753	548	72.8	36.08
3	119	1040	8.7	5.32	687	504	73.4	36.10
4	112	954	8.5	5.30	645	485	75.2	35.05
5	101	820	8.1	5.18	567	411	72.5	38.66
6	87	635	7.3	5.32	467	323	69.2	40.63
7	74	505	6.8	5.37	374	248	66.3	41.90
8	58	363	6.3	5.36	286	204	71.3	42.79
9	46	265	5.8	5.55	218	155	71.1	45.80
10	42	239	5.7	5.50	199	158	79.4	42.74
11	31	168	5.4	5.32	141	97	68.8	44.10

[1] Stock food.

number; 2) the average weight of rat pups soon after birth is fairly constant;[7] 3) heavier rats are raised as the average number per litter decreases.

[7] We cannot take issue with the finding of King ('24), that the weight of newborn rats is a function of the age of the mother inasmuch as our average weights usually include the weight of milk fro

SUMMARY

Data on ten stock and 135 experimental rats, receiving different levels and ratios of calcium and phosphorus, and 8431 young born to these mothers during eleven consecutive reproductive cycles have been compiled. It is noted that: 1) the rat is relatively cartilagenous at birth; 2) the mineral content of the rat at birth is relatively constant, in spite of wide differences in maternal mineral intake; 3) because of constancy in the composition of the body ash of 21-day-old rats, even when their mothers receive varying mineral intakes, the percentage of calcium and phosphorus in their gross bodies is calculable with fair accuracy from the percentage ash; 4) with the exception of high phosphorus diets, the body ash of sucklings (21 days old) may be used as a measure of bone ash, or calcification; 5) female rats, 21 days old, contain more ash, calcium, and phosphorus than do males of the same age; and 6) during consecutive reproductive cycles the number of young born per litter decreases, but the average weight at weaning increases.

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ENERGY AND GASEOUS METABOLISM OF NORMAL AND DEUTECTOMIZED CHICKS BETWEEN 10 HOURS AND 100 HOURS OF AGE [1]

H. G. BAROTT, T. C. BYERLY AND EMMA M. PRINGLE
Beltsville Research Center, Animal Husbandry Division, Bureau of Animal Industry, U. S. Department of Agriculture, Beltsville, Maryland

SEVEN FIGURES

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OBJECT OF INVESTIGATION

The object of this research was to determine 1) the energy metabolism of chicks of both sexes, both normal and deutectomized,[2] and 2) the effect of temperature of environment on the energy metabolism. Data showing the energy metabolism and the effects of environmental temperature on metabolism are necessary for the elucidation of numerous problems in the field of physiology and nutrition. "At present no question is more important in the physiology and genetics of sex than that of the relation which metabolism bears to sex" (Benedict and Riddle, '29). The effect of the temperature of environment on energy metabolism is of equal or greater importance. The recent summary and analysis of the data for heat production of chickens of both sexes and various ages by Mitchell and Kelley ('33) indicate a notable lack of data for newly hatched chicks.

Measurements of normal chicks under 5 days of age cannot be accepted as measurements of basal heat production since such chicks contain varying quantities of unabsorbed yolk.

[1] Acknowledgment is made to W. H. Burrows of the Animal Husbandry Division, who performed most of the deutectomies, and to Robert Rector, and J. L. Gardiner, also of the Animal Husbandry Division, for their assistance in carrying on the experiments and making the observations.

[2] Chicks from which the unabsorbed yolk has been surgically removed.

Consequently observations on chicks from which the unabsorbed yolk had been removed are necessary.

APPARATUS AND PROCEDURE USED

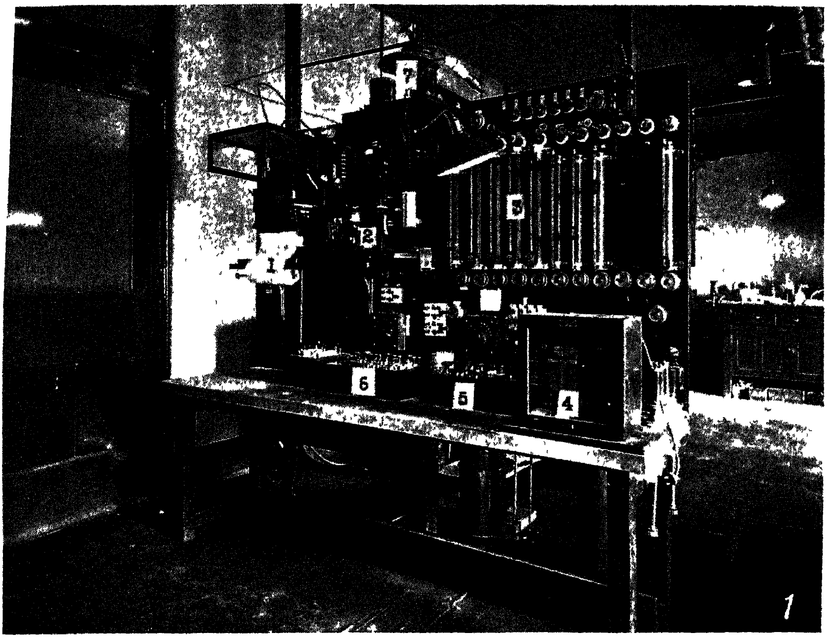
This investigation was conducted by the use of the respiration calorimeter, an instrument for the determination of heat elimination and gaseous exchange, with accurate control of all the physical factors involved. This instrument is described in detail by Barott[3] and by Langworthy and Milner ('15, '16).

The respiration calorimeter in the form used in this research is a precision instrument, the principles of which may be summarized as follows: for the determination of gaseous exchange the device is a gas-tight chamber connected to a system of gas absorbers in a closed circuit. The gas confined in the circuit is kept in circulation, the gaseous products imparted to it by the experimental birds are constantly removed and oxygen constantly supplied to replace that used. For the measurement of heat produced in the chamber the device is a constant-temperature, continuous water-flow calorimeter, in which the calorimetric features provide for preventing the passage of heat through the walls of the chamber or in the circulating air, and for taking up the heat by a current of water as fast as it is generated in the chamber. The determination of gaseous exchange and heat elimination, to be of value demands a high degree of accuracy in the fundamental measurements and it follows that the instruments with which they are made must be precise and finely adjusted. The calorimeter must be large enough for the purposes of the ex-

[3] Barott, H. G. Effect of temperature, humidity and other physical factors on the chick embryo during incubation. (In course of preparation.)

Fig. 1 Observation table and control board. 1, temperature recorder for water flow to absorber coil; 2, temperature controller for water flow to absorber coil; 3, rheostats to control boundary temperature; 4, controller to control boundary temperature; 5, potentiometer; 6, wheatstone bridge; 7, galvanometer system.

Fig. 2 Calorimetric assembly. 1, absorber coil; 2, absorption train for removing CO_2 and H_2O: a, H_2O absorber flasks; b, CO_2 absorber canister; 3, Crowell blower to provide gas flow through system; 4, standard wet gas meter for metering oxygen; 5, conductivity apparatus for gas analysis; 6, scale and can for weighing water from absorber coil.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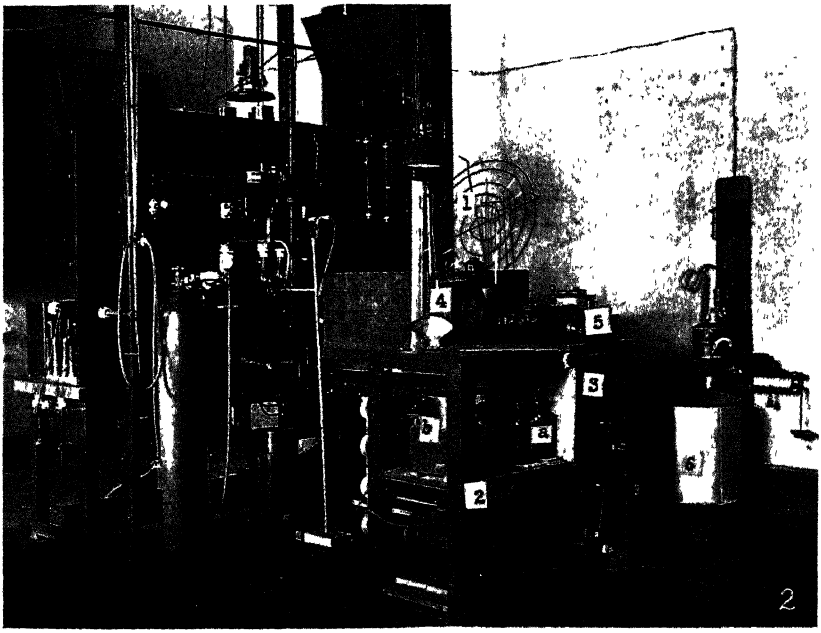
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periment and yet not so large that its volume will prevent the accurate measurement of the different factors involved. Its walls must be absolutely air-tight, because any leakage through them would nullify the determination of the gaseous exchange and there must be no passage of heat through them

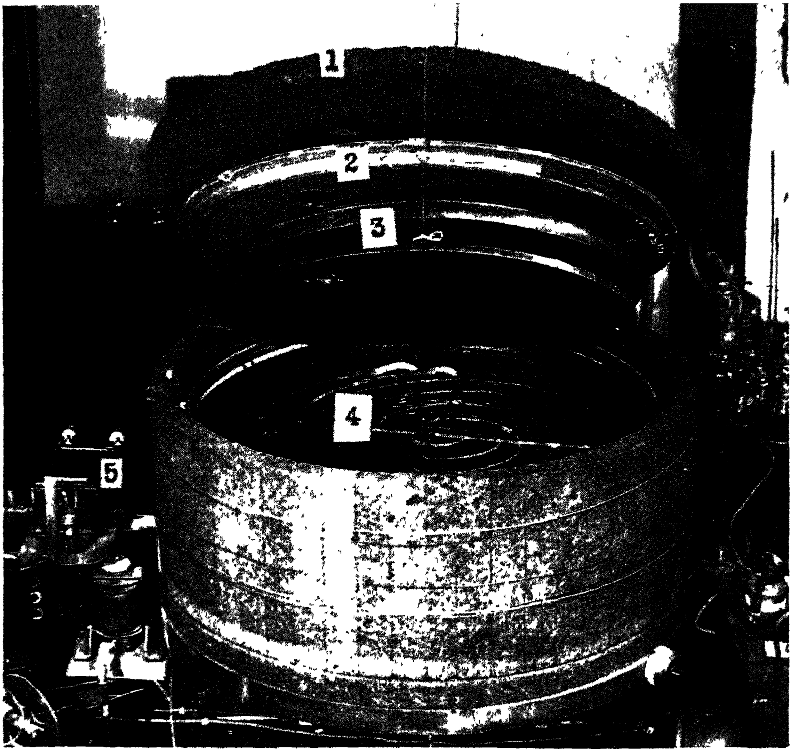

Fig. 3 Calorimeter. 1, outer cork cover; 2, heating coils for control of temperature of boundaries; 3, double copper covers; 4, absorber coil; 5, spirometer to regulate oxygen supply to tension equalizer.

because any transference of unmeasured heat into or out of the chamber would introduce an error into the determination of energy produced within it. Figures 1, 2 and 3 show the assembly of the apparatus.

The calorimeter consists essentially of a double-walled, copper chamber (fig. 3) in the form of a cylinder, 65 cm. in

diameter and 15 cm. deep. The walls are separated by an air space 1 cm. wide in which are inserted a system of differential thermopiles for determining the relative temperature of the two walls. The total volume is approximately 50 liters. The entire top is removable and is hermetically sealed with universal wax.

This double-walled chamber is surrounded by a third boundary of thin insulation board, with an air space 1 cm. thickness separating it from the calorimeter. In this air space are inserted three electric heating coils, uniformly distributed over the surface, one over the top, the second under the bottom and the third around the sides of the outer copper chamber. By means of these coils heat can be supplied to the air space to control the temperature of the outer copper wall. Cork board, 5 cm. thick, on all sides gives further protection against fluctuations in temperature.

GASEOUS EXCHANGE

This copper calorimeter comprises the respiratory chamber. This together with the pump and gas line for circulating the air, and the absorption train for removal of products of respiration, makes up the system, which is of the closed circuit type being completely isolated from the surrounding environment.

The atmosphere of the empty chamber contains oxygen, nitrogen, carbon dioxide and water vapor in proportions like that of ordinary air. When the birds are placed therein, this proportion begins to change because of the consumption of oxygen and the elimination of carbon dioxide and water vapor. The removal of this excess carbon dioxide and water vapor and the restoration of the oxygen used, in such a manner that the quantity may be accurately measured forms the basis of the determination of the respiratory exchange in the chamber. The movement of the air is fixed and constant, once the speed of the pump is adjusted, and the air is uniformly distributed, thus keeping the gas of uniform composition throughout the chamber.

The water vapor and carbon dioxide imparted to the air is removed in specially designed absorbers (fig. 2 a, b). The increase in weight of the absorbers in a given period shows the quantities of these products carried out of the chamber during this period. In a system of this type, as fast as any gas is removed from the air, other gas is introduced to maintain atmospheric pressure in the chamber; in this case, oxygen is supplied automatically. The quantity supplied is accurately metered for the period covering the measurements. The amount of water vapor, carbon dioxide and oxygen measured, corrected for the change in composition of the gas within the chamber, determines the quantity of these products. The amount of carbon dioxide and oxygen in the air of the system is determined by volumetric analysis by use of a Shepherd ('31) gas analysis apparatus; the amount of water vapor is computed from the relative humidity which is measured by a special hair hygrometer.

The volume of air in the chamber varies constantly with the admission of oxygen, the removal of carbon dioxide and water vapor, and with changes of temperature of the air in the chamber and barometric pressure outside. Although this variation is small in magnitude it could result in undesirable variations in the pressure of air in the chamber unless provisions were made for corresponding fluctuations in the capacity of the system. This is accomplished by admitting the oxygen to the chamber through a delicately balanced spirometer which also serves as a tension equalizer.

The absorption train is connected as a shunt off the main gas line, and the volume of air passing through the absorbers is so regulated as to keep the humidity and carbon dioxide content constant.

DETERMINATION OF HEAT PRODUCED

Heat is removed from the calorimeter in two ways: as latent heat of water vapor in the circulating air and as sensible heat liberated to the air by conduction and radiation from the birds. Both latent and sensible heat must be determined.

The water vaporized leaves the chamber in the outgoing air. The quantity of heat leaving the chamber as latent heat of water vapor in any given period is determined by multiplying the weight of water absorbed during the period by the latent heat of water.

The energy eliminated as sensible heat is absorbed by a current of water which circulates through the chamber in the heat absorber coil made of several turns of thin-walled copper tubing. The weight of the water in kilograms that flows through the absorber during a given period, multiplied by the difference of temperature of the water as it enters and leaves the chamber measured by platinum resistance thermometers and converted into degrees Centigrade, represents the amount of heat removed during the period expressed in calories at the mean temperature of the water flowing in the absorber coil.

The rate at which heat is removed from the calorimeter is regulated to prevent fluctuations in the temperature of the air in the chamber. By control of the temperature of the water as it enters the chamber and of the quantity of water which passes through the absorber coil, the removal of heat may be made to accord with its production in the chamber within very narrow limits.

The temperature of the water flowing to the absorber coil is automatically controlled by an electric water heater. The temperature of the walls of the copper chamber and the air within the chamber is determined by means of electric resistance thermometers and by various thermo-couples. The thermal relationship of the two copper walls is determined by a system of thermo-piles kept in equilibrium by the heating coils surrounding the outer wall. The relative temperature of the inflow and outflow air is determined by a differential thermo-pile, one junction of which is inserted in each line at the point of junction with the calorimeter. The temperature of the inflow air is maintained at the same temperature as the outflow by means of a water-cooled heat interchanger and an electric heating coil on the inflow air line.

TESTS FOR ACCURACY OF THE CALORIMETER

At frequent intervals, the accuracy with which measurements can be made with the respiration calorimeter is checked. These checks comprise tests of the apparatus as a calorimeter and as a respiration chamber. To check the instrument as a calorimeter, electric energy is converted into heat in a resistance coil within the chamber, and this heat is measured calorimetrically. The amount produced in a given period of time can be accurately determined from measurements of current flowing in the coil and the voltage drop across the coil. The calorimetric measurements check the electrical to an accuracy of 1 per cent, or in the measurements of very small amounts of energy, by one small calorie.

To check the accuracy of the apparatus as a respiratory chamber, ethyl alcohol is burned in the chamber in such a manner as to insure complete combustion, and the products of combustion consisting of water vapor and carbon dioxide are absorbed and measured. The oxygen used is also measured. These measured amounts are compared with the theoretical amounts computed from the chemical equation for the reactions occurring in the combustion of ethyl alcohol. The results checked to an accuracy of better than 0.5 per cent when 4 gm. of alcohol per hour were burned.

CHICKS USED AND EXPERIMETAL PROCEDURE

The chicks used were of mixed breeding and were obtained from breeding pens kept at the United States Department of Agriculture Research Center, Beltsville, Maryland.

The chicks were placed in wire net boxes with covers. Each box was 5 × 8 × 3 inches in size and was divided into three compartments, each of which accommodated two or three chicks comfortably. The amount of space allowed each chick prevented huddling and also much physical movement.

The effect of temperature variation on energy metabolism was determined at temperatures from 64° to 107°F., with normal and from 68° to 107°F. for deutectomized chicks of both sexes.

For the sex studies normal chicks from one hatch were divided into two lots, one male and one female. The sex was determined by visual examination of the copulatory organs according to the method reported by Jull ('34). After the experiment was completed the chicks were killed for the purpose of verifying the sex. The visual method was shown to have an accuracy of more than 90 per cent. The two groups were placed alternately in the calorimeter for 8-hour periods from the time of sexing to the time when they had reached an age of from 60 to 100 hours. The relative humidity was kept at 60 per cent, the carbon dioxide at less than 1 per cent, and the oxygen at 21 per cent during the observational periods. Between observational periods the chicks were kept in commercial chick boxes at room temperature, the usual manner of holding chicks of their age. Complete measurements were made each 4 hours of the 8-hour period, thus giving two determinations. The chicks were weighed immediately before and after each 8-hour period.

For the experiments involving deutectomized chicks, the unabsorbed yolk in the chick's body at hatching time was readily removed by a simple operation, as reported by Sloan, Card and Adamstone ('34). All deutectomized chicks were allowed at least 8 hours for recovery before being placed in the calorimeter. A few chicks died of hemorrhage within the first 2 hours after operation. The fact that the gram hour rate of metabolism of the operated chicks was proportionately lower than that of the unoperated chicks and that it subsequently dropped regularly with time, is the only evidence available that the recovery period was sufficiently long. The appearance and behavior of the operated chicks was quite normal.

The chicks were divided into three lots: males with yolk sac removed, females with yolk sac removed and normal males used as controls. This gave comparative studies of normal males which have the unabsorbed yolk to live on, and deutectomized males which were deprived at once of all food supply, and a comparison of sexes among deutectomized chicks.

These were placed in turn in the calorimeter beginning with the males used as controls, for 12-hour observational periods. Complete measurements of total heat and gaseous exchange were made every 4 hours during the 12-hour period until the chicks reached an age of from 60 to 100 hours. No feed or water was given at any time after hatch. The chicks were weighed immediately before and after each 12-hour period.

RESULTS FOR NORMAL CHICKS

Figure 4 gives the results on heat elimination and gaseous exchange for normal chicks, in the form of graphs. The results on the normal males used as controls for the deutectomized chicks are added to complete the data obtained. Each point represents the mean value of from four to twelve observations using from twenty-two to thirty-two birds. The effect of variation of temperature is very pronounced. The minimum values occur at approximately 96°F. At this temperature, therefore, is the least expenditure of energy and consequently less food required for basal upkeep.

Mitchell and Haines ('27), reported the critical temperature for Rhode Island Red hens to be about 62°F. but an analysis of their data by the authors shows it to be not lower than 70°F. The critical temperature for the chicks used, under the conditions of the present experiments, was 96°F.

As the temperature varies in either direction the energy metabolism becomes greater until at 89° and 101°F. it has increased nearly 15 per cent. Below 89° the increase is proportional to the decrease in temperature until a temperature of 70°F. is reached, when it is nearly twice as great as at 96°F.

The newly hatched chick is unable to compensate for environmental temperature lower than 70°F. by increasing its own heat production. Chicks permitted to huddle could undoubtedly withstand somewhat lower temperatures.

There is also an appreciable increase in the metabolic rate at temperatures higher than 96°F. At 104°, as the body temperature of the chick is approached, the chick is no longer

able to compensate for high temperatures and the energy metabolism approaches a constant value above this temperature, within the range of temperature studied.

The results in figure 4, which are paired (male and female), were obtained on the same day, one test following the other immediately. All conditions for the two tests were as nearly

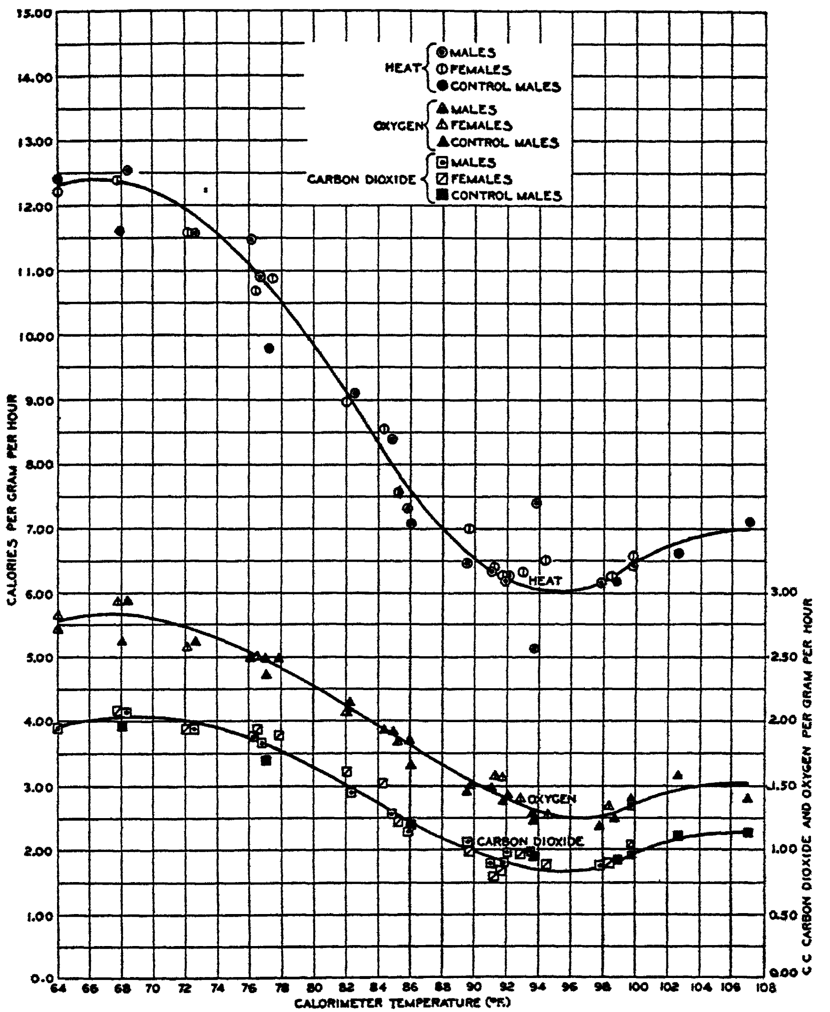

Fig. 4 Heat elimination and gaseous exchange for normal chicks 10 to 100 hours of age.

identical as it was possible to get them. Day and night periods were equally divided among observations on male and female chicks so that the data are not biased by diurnal variations. No consistent difference in energy metabolism between the sexes is shown by these results.

RESULTS FOR DEUTECTOMIZED CHICKS

The heat elimination and gaseous exchange for normal chicks remains practically constant at a given temperature for the first 4 days, but with deutectomized chicks the values for these quantities decrease in direct proportion to the time elapsed after operation.

The graphs, figure 5, show this rate of change, from the time of operation, for each temperature. The values for males and for females are plotted as separate points and each point represents the mean of from three to eight independent observations.

The point for the zero hour on figure 5 is the value for normal chicks at that temperature. If we take the slope of the graph for each temperature in terms of per cent of the normal value (the value at the zero hour) and use this proportionality factor, the values for energy metabolism for deutectomized chicks may be computed from the values for normal chicks.

Values for this factor were computed for each of the temperatures studied and these plotted on figure 6.

The change in metabolic rate with time at the temperatures studied is greatest at 68°F. and decreases with temperature at practically a constant rate.

The metabolism of the chick is greatest at low temperatures, decreases to a minimum at 96°F. and then increases again, which is the same as observed for normal chicks.

Values from the graph, figure 6, incorporated in the following equation will give values for deutectomized chicks in terms of data from normal chicks.

$$Q_d = Q_n at$$

where Q_d is the number of $\left\{\begin{matrix}\text{calories heat}\\ \text{cubic centimeter } CO_2\\ \text{cubic centimeter } O_2\end{matrix}\right\}$ per hour per gram weight for deutectomized chicks. Q_n is the number of $\left\{\begin{matrix}\text{calories heat}\\ \text{cubic centimeter } CO_2\\ \text{cubic centimeter } O_2\end{matrix}\right\}$ per hour per gram weight of normal chicks.

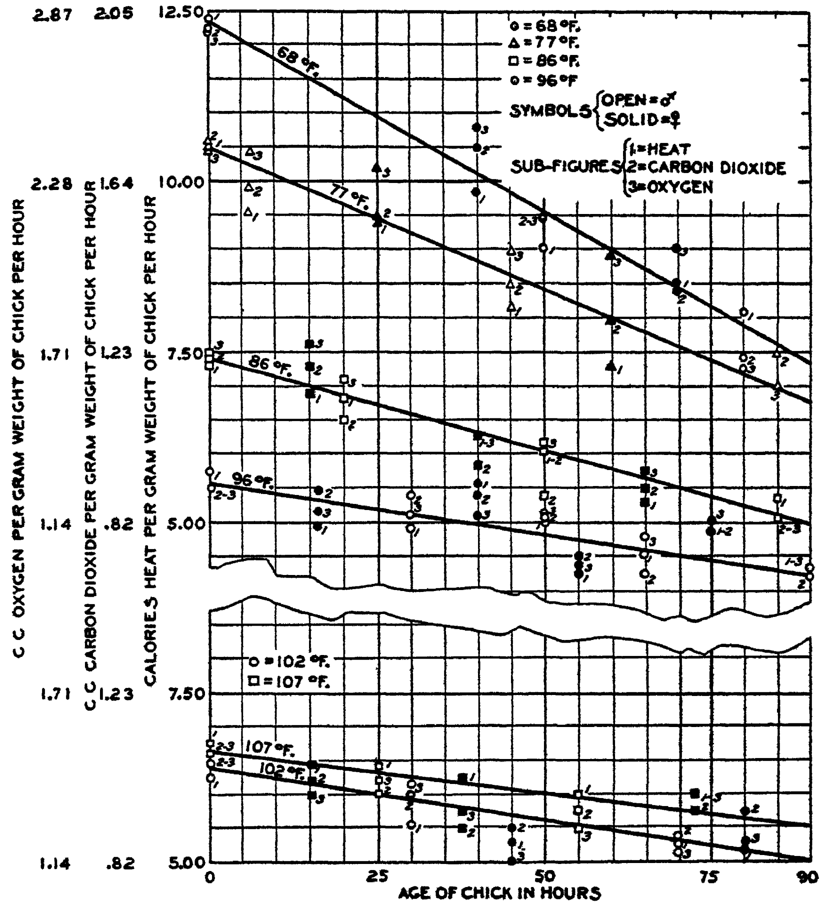

Fig. 5 Energy metabolism of deutectomized chicks.

a is the proportionality factor from the graph, figure 6, and *t* is the time in hours after operation.

The values, figure 6, apply only to chicks which are exposed 12 hours to the experimental environment, then 24 hours to

brooder environment, followed by another 12 hours to experimental environment, etc.

Values for heat, carbon dioxide, and oxygen for deutectomized chicks may be computed from the above formula. A

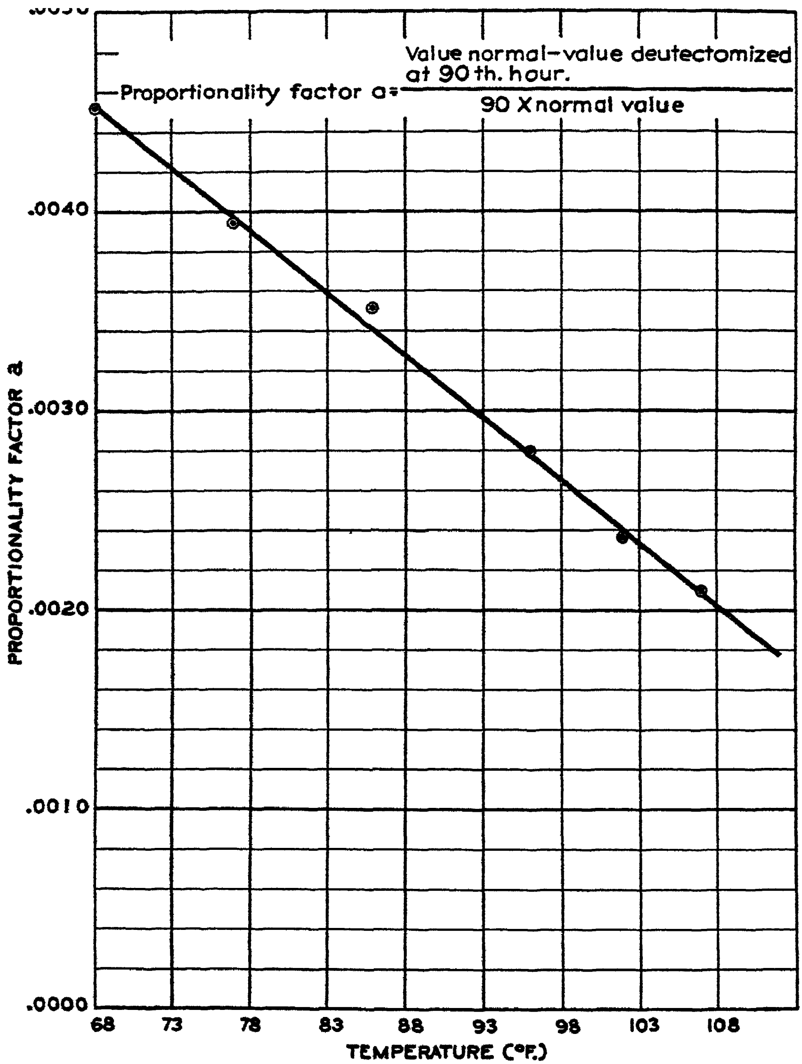

Fig. 6 Graph for computation of values for deutectomized chicks from values for normal.

graph plotted with temperature as abscissa and these values as ordinates will follow that for normal chicks in general characteristics, but each value will of course be less than that for normal chicks by an amount dependent upon the time elapsed after operation.

The graphs, figure 5, for deutectomized chicks show points for males and for females. The position of the points relative to the graph gives an indication of the relative values with respect to sex. All data considered show no difference in energy metabolism due to sex for the first 4 days after hatch.

Benedict, Landauer and Fox ('32), and Mitchell, Card and Haines ('27), have obtained data for older birds where no difference in metabolism between the sexes was apparent when expressed in terms of body weight.

RESPIRATORY AND THERMAL QUOTIENT

The respiratory quotient, cubic centimeter of CO_2 divided by cubic centimeter of O_2, and the thermal quotient, calories heat divided by grams of CO_2, for normal chicks, computed from the results of this investigation, are 0.71 and 3.16 respectively, with no consistent variation with temperature. Benedict, Landauer and Fox ('32), and Kleiber and Dougherty ('34), have found comparable values for older birds. This indicates practically no carbohydrate metabolism. The respiratory quotient is that for fat or protein metabolism which gives the same R. Q. when the end product of protein metabolism is uric acid as in the chick, as was shown by Henry, Magee and Reid ('34). The thermal quotient for fat should give 3.37, so here also is an indication of some protein metabolism which would have a tendency to lower the thermal quotient below the 3.37 value.

The respiratory quotient and the thermal quotient for the deutectomized chicks are the same as for the normal chicks.

LOSS IN WEIGHT OF CHICKS

The rate at which the chick lost weight under the experimental procedure was constant relative to time. It was also

constant relative to temperature below 100°F. except for a slight increase below 80°F., but above 100°F. the rate of loss increased rapidly. This was due, no doubt, to excessive loss of water because of the chick's effort to maintain its body temperature by evaporation when the environmental temperature was approaching its body temperature.

The loss in weight in 3 days in per cent of original weight is as follows:

TEMPERATURE CALORIMETER	NORMAL CHICKS	NORMAL CHICKS LESS CORRECTION FOR YOLK SAC[1]	DEUTECTOMIZED CHICKS
°F.	*per cent*	*per cent*	*per cent*
65–80	23.5	9.0	26.0
80–100	21.5	9.0	22.0
102	31.0	16.5	28.5
107	42.0	29.5	38.0

[1] The average weight of the normal chicks at the beginning of the experiments was 35.9 gm. and that of the deutectomized chicks 30.5 gm. Thus the average weight of the yolk sac removed was approximately 5 gm. Subtracting this amount from the loss in weight of the normal chick will give the weight of body tissue which was used. This is given as a per cent of the original weight in the above tabulation.

HEAT ELIMINATION AND GASEOUS EXCHANGE PER GRAM LOSS IN WEIGHT OF CHICKS

Figure 7 shows by graphs the heat, carbon dioxide, and oxygen per gram loss in weight of chick for normal chicks for the 3-day period over the range of temperature studied.

The values for gram loss of weight for deutectomized chicks could be computed for any specified time but the values would become lower and lower with time elapsed after operation. They would in all cases be lower than those for normal chicks.

Figure 7 shows the greater efficiency in utilization of body material at the lower temperatures. The heat production per gram loss in weight at 68°F. is more than twice as great as that at 107°F.

The peak in the graph at 98°F. is interesting. The weight loss below 100°F. is very nearly constant, but the energy output at 98° is about 5 per cent greater than at 94°, which causes the graph to rise at this interval. Above 100° the

energy output increases slightly but the rate of loss in weight of the chick increases rapidly thus causing the graph to fall once more.

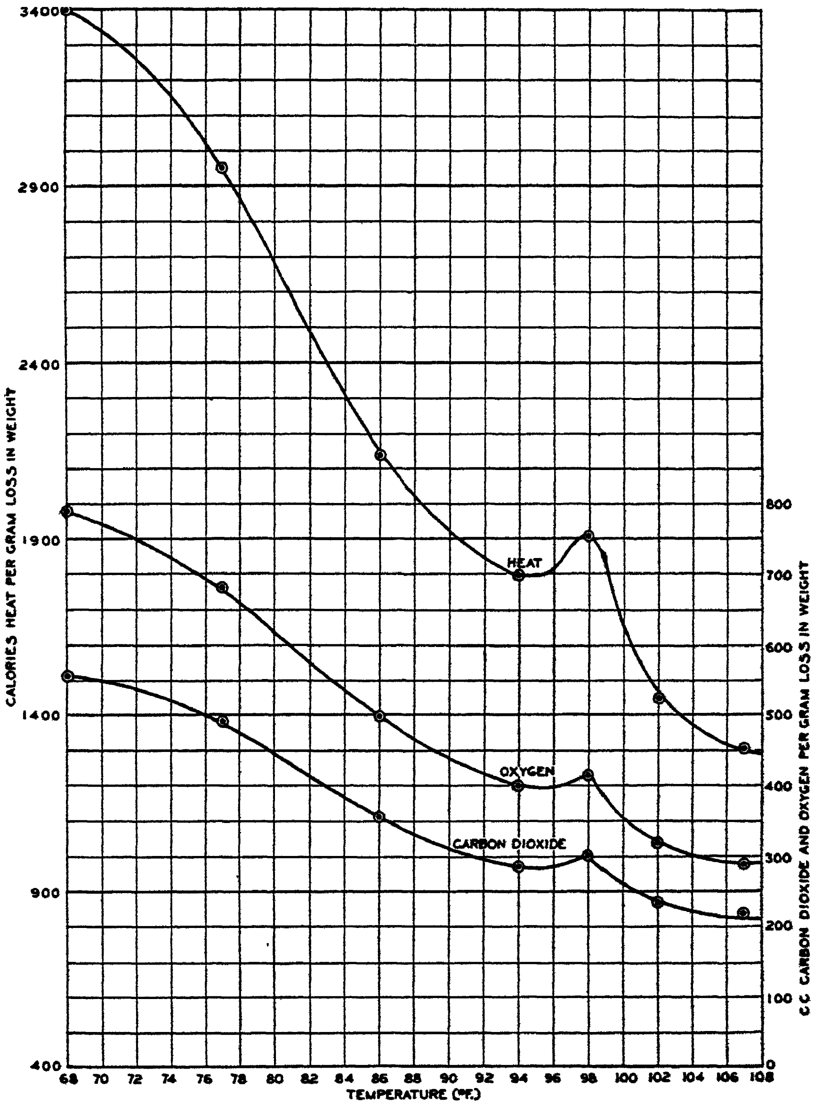

Fig. 7 Heat elimination and gaseous exchange of normal chicks per gram loss in weight.

The data for oxygen consumption of newly hatched chicks presented by Mitchell, Card and Haines ('27), check within 10 per cent those in this paper, at points approximating the critical temperature.

Heywang and Jull ('30) have shown that the yolk in young chicks is not totally absorbed until the seventh day after hatch, when chicks are fed. They have also shown that the yolk absorption the first 72 hours after hatch is the same whether birds are fed or unfed. Byerly (personal communication) has extended the observations on unfed chicks to the sixth day and finds that the yolk absorption is practically the same for fed or unfed chicks but that the fed chicks gain more in true body weight than the unfed.

The constancy of energy metabolism of normal chicks during the first 4 days in this investigation would be expected from this fact relative to yolk absorption together with the fact that the environment of the chicks during this investigation was uniform.

The data on deutectomized chicks showing a continuous and uniform decrease in metabolism for the first 4 days illustrate the necessity, in all cases where basal values for metabolism are stated, to comply closely with the definition for basal values, i.e., the energy exchange which is sufficient to maintain normal functions when activity of the organs is diminished as far as possible; in practice where the bodily activity is reduced to a minimum and measured 12 to 18 hours after eating a light meal.

The close similarity of the values obtained for the two sexes at the critical temperature has been found by previous workers for male and female chicks of the same body weights (compare Mitchell and Kelley, '33). The critical temperature for newly hatched chicks is much higher than that reported by Mitchell and Haines ('27) for hens. This may be due to the relatively greater surface area in proportion to mass of the chicks.

The response of male and female chicks to temperature change is unlike that reported by Riddle, Christman and

Benedict ('30), for ring doves, for their data showed much greater metabolism of the male at lower temperatures than of the female. In the present experiments, values for males and females at all temperatures showed neither appreciable nor consistent differences.

The average decrease in heat production caused by shifting male ring doves from an environmental temperature of 20°C. to one of 30°C. was 28.1 per cent and for females 20.3 per cent, while the male chicks here reported showed a decrease of 39.5 per cent and the females 40.0 per cent.

SUMMARY AND CONCLUSIONS

Heat production and gaseous exchage were determined for normal and deutectomized chicks of both sexes for the period from 10 to 100 hours of age by direct calorimetry. Chicks were studied at temperatures from 68 to 104°F., inclusive.

The temperature of environment showed a decided effect on the metabolism. The chicks displayed a critical temperature at 96°F. Seven degrees increase or decrease from the critical temperature caused about 15 per cent increase in metabolism. With decrease in temperature from 96 to 70°F., metabolism increased in proportion to environmental temperature until at 70°F. the energy output was twice as great as at 96°F. The chick was unable to compensate for temperatures below 70°.

Neither appreciable nor consistent differences were found between chicks of the two sexes at any temperature studied.

The R. Q. observed was about 0.71 and the T. Q., calculated from CO_2, 3.16, which indicates a metabolism free of carbohydrate.

The gram hour rate of metabolism for normal chicks is constant at any given temperature within the physiological range for the age period studied. The gram hour rate for deutectomized chicks decreased continuously and in direct proportion to time elapsed after operation.

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CALCIUM AND PHOSPHORUS RETENTION IN GROWTH, IN RELATION TO THE FORM OF CARBOHYDRATE IN THE FOOD

MARY SPEIRS AND H. C. SHERMAN
Department of Chemistry, Columbia University, New York City

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Without asking space to review the voluminous literature either of carbohydrate digestion and metabolism or of the economy of calcium and phosphorus in nutrition, we would here record the results of experiments upon calcium and phosphorus retention, in the growing body, when the food, alike in other respects, was systematically varied as to the nature of the carbohydrate which constituted one of the major components of the dietary. The forms of carbohydrate here compared are dextrose (corn sugar, 'cerelose'), spray-dried corn sirup (mixture of dextrose, maltose, and dextrin), dextrin (a commercial edible dextrin made from cornstarch), cornstarch, and sucrose. A series of diets was prepared in each of which one of the dry forms of carbohydrate just named was mixed with twice its weight of the air-dry diet B (diet 13) which has been extensively used in previous investigations in this laboratory (Sherman and Campbell, '24; Sherman and MacLeod, '25; Sherman and Quinn, '26; Sherman and Booher, '31; Fincke and Sherman, '35).

It is known that this diet B, when given alone, supports normal growth and development. Hence its dilution with one-half its weight of carbohydrate was believed to furnish a favorable means of comparing different forms of carbohydrate as to their effect upon calcium and phosphorus retention by the growing and developing organism, especially

if the experimental period were chosen as falling in a segment of the life cycle in which normal development includes a considerable retention of calcium and phosphorus as an essential feature of the normal calcification or ossification of the skeletal system.

EXPERIMENTAL

Young albino rats were transferred at 28 to 29 days of age from the family diet of one-third dried whole milk with two-thirds ground whole wheat (diet B; laboratory no. 13) to experimental diets consisting of two-thirds diet B and one-third of the form of carbohydrate under investigation—dextrose, spray-dried corn sirup, dextrin, cornstarch, or sucrose, respectively—litter-mate controls being continued upon diet B. At 60 days of age, the experimental animals including the controls were killed and analyzed for calcium and phosphorus. The age of 60 days was chosen for this purpose because previous experience of our laboratory with rats of the same colony and nutritional background indicated this to be the age at which the determination would be most conclusive as to whether, on these different experimental diets, calcium (and phosphorus) retention would be alike or different. The data of calcium and phosphorus contents of duplicate rats at the age of 28 to 29 days, as determined both in these experiments and in much related work in this laboratory, make it possible for us to compare the diets characterized by the different forms of carbohydrate here studied, both in terms of the calcium and phosphorus contents of the experimental animals at the age of 60 days and in terms of the amounts of these elements which had been retained during the experimental period.

Calcium was determined by the well-known modified McCrudden method, and phosphorus according to the method of the Association of Official Agricultural Chemists.

In starting the experiments, rats of the same litter were distributed to the different diets in such a way as to afford the fullest practicable measure of litter-mate control in the

comparison of the results obtained upon the different experimental diets with each other, and with those obtained on the control diet (diet B); also to have as even as practicable a distribution of sexes and initial body weights.

The forms of carbohydrate here studied contained from no detectable traces to barely measurable amounts of calcium and phosphorus. Hence all experimental diets contained 0.23 to 0.24 per cent of calcium and 0.32 to 0.33 per cent of phosphorus with a calcium: phosphorus ratio of 0.7. In the light of the previous experience of our laboratory, this appears a sufficiently favorable intake and ratio to constitute a normal nutritional test, yet with the level of calcium intake low enough so as really to put to the test any difference which might exist among the diets containing the different forms of carbohydrate. That such was the case may be seen from the fact that this dilution of the control diet with the carbohydrate studied did result in every case in a slightly less rapid retention of calcium (and phosphorus) than occurred in the control animals which received the undiluted diet B with its 0.35 per cent of calcium and 0.49 per cent of phosphorus and with the same calcium: phosphorus ratio of 0.7. Records of food consumption during the experimental period permit also the approximate estimation of the calcium and phosphorus intakes. The data of these experiments are summarized, for calcium and for phosphorus, respectively in tables 1 and 2.

Rats here analyzed at 28 to 29 days of age showed: for males, 0.68 ± 0.03 per cent calcium and 0.51 ± 0.02 per cent phosphorus; for females, 0.71 ± 0.03 per cent calcium and 0.53 ± 0.02 per cent phosphorus (on the basis of live weight). These percentages are in agreement with the previous experience of this laboratory in like cases, account being taken of the fact that the percentages are expressed sometimes on the basis of live weight and sometimes of net weight as above explained.

TABLE 1

Calcium retentions by rats on diets containing different carbohydrates

ADDED CARBOHYDRATES IN DIET	NUMBER OF RATS	CALCIUM IN FOOD EATEN	NET WEIGHT AT 60 TO 61 DAYS	CALCIUM IN BODY AT 60 DAYS		CALCIUM RETAINED DURING EXPERIMENTAL PERIOD
Males						
		gm.	*gm.*	*gm.*	*per cent of net weight*	*gm.*
Control diet	5	1.077±0.096	148±19	1.169±0.129	0.79±0.02	0.829±0.113
Dextrose	3	0.728±0.066	126±18	0.957±0.153	0.76±0.02	0.613±0.105
Dried corn syrup	4	0.718±0.067	124±10	0.973±0.077	0.79±0.02	0.638±0.061
Dextrin	4	0.661±0.026	114± 8	0.893±0.075	0.79±0.02	0.558±0.046
Cornstarch	3	0.705±0.053	124± 6	0.985±0.051	0.80±0.02	0.625±0.030
Sucrose	4	0.729±0.039	128± 9	0.954±0.054	0.75±0.02	0.629±0.026
Females						
Control diet	4	0.922±0.018	116± 1	1.036±0.014	0.90±0.01	0.708±0.017
Dextrose	3	0.605±0.046	99± 4	0.822±0.027	0.83±0.01	0.509±0.019
Dried corn syrup	5	0.671±0.024	110± 8	0.917±0.051	0.83±0.03	0.574±0.026
Dextrin	3	0.597±0.057	105±10	0.901±0.089	0.86±0.02	0.539±0.065
Cornstarch	3	0.590±0.014	105± 8	0.886±0.072	0.85±0.02	0.530±0.028
Sucrose	4	0.644±0.025	106± 4	0.939±0.053	0.89±0.01	0.581±0.032

TABLE 2

Phosphorus retentions by rats on diets containing different carbohydrates

ADDED CARBOHYDRATES IN DIET	NUMBER OF RATS	PHOSPHORUS IN FOOD EATEN	NET WEIGHT AT 60 TO 61 DAYS	PHOSPHORUS IN BODY AT 60 DAYS		PHOSPHORUS RETAINED DURING EXPERIMENTAL PERIOD
Males						
		gm.	*gm.*	*gm.*	*per cent of net weight*	*gm.*
Control diet	5	1.499±0.125	148±19	0.813±0.116	0.55±0.03	0.558±0.101
Dextrose	3	1.008±0.091	126±18	0.664±0.077	0.53±0.01	0.406±0.042
Dried corn syrup	4	0.970±0.091	124±10	0.691±0.057	0.56±0.01	0.440±0.041
Dextrin	4	0.921±0.041	114± 8	0.633±0.052	0.56±0.01	0.381±0.030
Cornstarch	3	0.976±0.074	124± 6	0.698±0.039	0.57±0.02	0.427±0.022
Sucrose	4	1.010±0.054	128± 9	0.688±0.044	0.54±0.01	0.444±0.023
Females						
Control diet	4	1.290±0.025	116± 1	0.712±0.009	0.62±0.01	0.466±0.011
Dextrose	3	0.843±0.060	99± 4	0.578±0.022	0.58±0.00	0.342±0.012
Dried corn syrup	5	0.906±0.032	110± 8	0.644±0.038	0.58±0.02	0.389±0.020
Dextrin	3	0.827±0.078	105±10	0.633±0.063	0.60±0.01	0.362±0.045
Cornstarch	3	0.816±0.020	105± 8	0.616±0.042	0.59±0.01	0.360±0.017
Sucrose	4	0.913±0.016	106± 4	0.677±0.055	0.63±0.02	0.407±0.034

DISCUSSION OF RESULTS

As contemplated in the plan of investigation above explained, the animals which received the undiluted diet B—the control diet in this case—retained more calcium and phosphorus than those on the experimental diets. Hence it is logical to infer that the experimental diets were such as to constitute a real test of the question whether calcium (and phosphorus) retention procedes with similar or with different efficiency upon diets containing the different carbohydrates here studied.

Also, the animals on the control diet grew somewhat more rapidly than those in the experimental diets, since in the latter the concentration of tissue-building nutrients was diluted by the added carbohydrate. This dilution was, however, quantitatively the same in all cases, so that all these experimental diets contained the same concentrations and ratios; of calcium and phosphorus, and of all other tissue-building nutrients as well, and all were of essentially the same energy or calorie value per gram.

Moreover, the animals receiving the experimental diets grew at rates within the normal range for animals of their age and initial size, and the gains in body weight were essentially alike for all the experimental diets.

The gains in body calcium were also essentially alike on all the experimental rations; as were also the gains in body phosphorus. The small differences in the averages for either calcium or phosphorus retention for animals of either sex on any of the experimental diets appear to lie within the range of probable experimental error. Apparently the retention of calcium (and also of phosphorus) proceded with essentially equal efficiency in the normal growing (mammalian) body whether the dietary (otherwise composed of wheat and milk) contained dextrose (corn sugar, 'cerelose'), spray-dried corn sirup, dextrin, cornstarch, or sucrose as added carbohydrate.

Moreover, the percentages of calcium (and of phosphorus) in the bodies of the animals at the end of the experimental period were essentially alike for those on the control diet and those on the diets diluted with the different carbohydrates.

As would be expected from the greater richness of the control diet in calcium, the animals on this diet used their ingested calcium a little less efficiently than did those on the experimental diets. To compare further the efficiency of utilization on the different experimental diets, the calcium retained divided by the calcium ingested may be shown as a 'calcium utilization factor' as in table 3. Here again the calcium metabolism is seen to be essentially alike under the influence of the different carbohydrates tested, as the small

TABLE 3

'Calcium utilization factors' (ratio of retained to ingested calcium) and ratios of body calcium to body phosphorus as indicated by analyses of experimental animals at 60 to 61 days of age

ANIMALS FROM DIET CONTAINING	MALES		FEMALES	
	Calcium utilization factor	Ca : P ratio	Calcium utilization factor	Ca : P ratio
Dextrose	0.83±0.06[1]	1.4	0.84±0.04	1.4
Dried corn syrup	0.89±0.04	1.4	0.85±0.04	1.4
Dextrin	0.84±0.04	1.4	0.90±0.03	1.4
Cornstarch	0.89±0.03	1.4	0.90±0.02	1.4
Sucrose	0.87±0.05	1.4	0.90±0.02	1.4

[1] Average deviation.

apparent differences in utilization factor are all within the range of experimental error—furthermore the animals on the different diets show the same ratio of body calcium to body phosphorus. Undoubtedly phosphorus retention was here determined by the experimentally restricted intake of calcium which was the limiting factor in the calcification of the growing bodies.

SUMMARY

The experiments here reported were planned to ascertain whether calcium and phosphorus retentions in the normal, growing young mammal are essentially alike or different when different forms of carbohydrate are added to an otherwise identical adequate diet.

In order that the experiments should constitute a sufficiently rigorous test of the question, each form of carbohydrate studied was mixed with twice its weight of the control diet (diet B or diet 13 of this laboratory, a mixture of two-thirds ground whole wheat and one-third dried whole milk; fed with table salt and distilled water). Thus in the experimental diets the tissue-building nutrients were diluted in all cases to the same extent; and to such extent as to make these diets somewhat less than optimal though still adequate to support body growth and skeletal development within the normal range.

Under these conditions it was found that calcium retention (and also that phosphorus retention) was essentially alike whether the carbohydrate was added to the diet in the form of dextrose (corn sugar), spray-dried corn sirup (mixture of dextrose, maltose and dextrin), dextrin, cornstarch or sucrose.

The experimental animals were albino rats bred in the laboratory. Their ages were exactly known, as were the nutritional histories both of the experimental animals and the families from which they came. The periods of experimental feeding began at the age of 4 weeks and were ended by killing and analyzing the animals at the age of 60 days, at which age any difference in calcification, which might have resulted from the difference in form of carbohydrate fed, should have been most readily demonstrable.

Within the limits of experimental error, calcium utilization was alike on the experimental diets containing the different forms of carbohydrate.

The ratio of calcium to phosphorus found in the body was also essentially the same for the animals which had received the different experimental diets.

Moreover, although growth and calcium retention were (designedly, as explained above) somewhat less rapid on the experimental diets than on the control diet, the percentages of calcium and of phosphorus in the bodies at the completion of the test were essentially the same for the animals on the experimental diets as for those of the same age on the control diet.

This latter fact constitutes added evidence that the level of calcium intake provided in the experimental periods was well adapted to the purpose of affording a rigorous test of the efficiency of the normal retention of calcium and phosphorus under the influence of these different diets.

We are indebted to the Corn Industries Research Foundation for aid in this investigation.

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THE EFFECT OF HEAT AS USED IN THE EXTRACTION OF SOY BEAN OIL UPON THE NUTRITIVE VALUE OF THE PROTEIN OF SOY BEAN OIL MEAL[1]

J. W. HAYWARD, H. STEENBOCK AND G. BOHSTEDT
Departments of Agricultural Chemistry and Animal Husbandry, University of Wisconsin, Madison

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Several investigators have reported that the use of heat in the preparation of certain foods definitely alters the nutritive value of the protein. When such materials as casein, meat, liver, kidney, heart muscle, cereals and fish meal were exposed to high temperatures for a considerable time, there resulted a decided decrease in their nutritive value. The reader is referred to a publication by Seegers and Mattill ('35) and by Boas Fixsen ('35) for a discussion of recent studies.

In direct contrast with these results, there is evidence that heating soy beans causes a definite improvement in the growth of animals when they are changed from a raw soy bean diet to a heated soy bean diet. Osborne and Mendel ('17), Vestal and Shrewsbury ('32), and Shrewsbury, Vestal and Hauge ('32) found that ground raw soy beans when fed to rats as the sole or principal source of protein in an otherwise complete ration did not support appreciable growth. However, normal growth resulted when they fed soy beans which had been previously cooked. Vestal and Shrewsbury ('32),

[1] This research was made possible by a fellowship supported by Allied Mills, Inc., Chicago, Illinois, to whom we want to express our indebtedness and appreciation. Published with the permission of the director of the Wisconsin Agricultural Experiment Station, Madison.

Shrewsbury, Vestal and Hauge ('32), and Robison ('32) reported similar results with pigs. Mitchell and Villegas ('23), Mitchell and Smuts ('32), McCollum, Simmonds and Parsons ('21), and Shrewsbury and Bratzler ('33) reported experimental evidence in support of the fact that the raw soy bean contains a protein of low nutritive value.

In an attempt to find the reason for the increase in growth brought about by heating the soy bean, Osborne and Mendel ('17) and Vestal and Shrewsbury ('32) conducted digestibility experiments with rats. Both groups of investigators reported that cooking the ground raw soy beans increased the digestibility of the total protein about 4 per cent. They stated that the improved nutritive value of the soy bean caused by heating appeared to be due to an increase in food consumption and nitrogen absorption.

Johns and Finks ('20) working with phaseolin, an isolated protein of the navy bean, found that this protein when cooked, produced markedly better growth, than the raw protein, in the rat. A similar improvement in the growth of the rat resulted when the isolated raw proteins of the velvet bean were fed cooked (Finks and Johns, '21). Cooked phaseolin (Waterman and Johns, '21) and the cooked isolated proteins of the velvet beans (Waterman and Jones, '21) yielded considerable more amino nitrogen when digested in vitro than the raw proteins digested in the same manner.

Although it is common knowledge that heat is used in all the commercial methods of extracting oil from the soy bean, little attention has been given to the effect that the amount of heating used in the process of extraction has upon the nutritive value of the protein in the residue known as soy bean oil meal. With the ever increasing demand for the production of soy bean oil, there arises the problem of using the soy bean oil meal to advantage (Horvath, '33). Apparently, as this meal is destined to be used extensively in rations for livestock and in the dietary of man, information on the effect that the oil extraction temperature has upon the nutritive value of the proteins of soy bean oil meal is important. For

the most part, two methods have been generally used for arriving at protein values; viz., the growth method of Osborne, Mendel and Ferry ('19) and the nitrogen balance method of Mitchell ('24). Since the two values are not comparable (Boas Fixsen, '35), we have employed both methods in the following experiments.

EXPERIMENTAL

Production of experimental material. Commercially, soy bean oil is extracted by the expeller, the hydraulic or the solvent processes. The resulting meals are known according to the method of extraction employed. In addition the hydraulic meal is sometimes spoken of as 'old process meal' and the solvent meal as the 'new process meal.' We included in our experiments these various soy bean oil meals, produced where possible at low, medium, and high temperatures. For the most part they were prepared from beans of the Illini variety. We supervised the production of the samples by the manufacturers and kept a complete history of the various treatments used in their preparation. The data pertaining to the temperatures used are given in table 1. In tables following, the beans used in preparing the expeller meals were designated with the letter A, those used for the hydraulic meals with B, and those used for the solvent meals with C.

Growth experiments. For the growth experiments the ground beans or meal were fed in a basal ration composed of about 50 parts cooked starch, 6 parts yeast, 4 parts of salts no. 40 (Steenbock and Nelson, '23) and 2 parts of cod liver oil. The amount of cooked starch incorporated into each ration was changed with the amount of soy bean material or other supplement required by analysis to supply the desired level of protein. Allowance was also made for any amount of soy bean oil or other ingredients other than the protein supplements specified in the rations.

We employed the ad libitum and equalized types of feeding (Steenbock, Black and Thomas, '30). We used male rats

3 to 4 weeks old at 50 to 60 gm. in weight. They were quartered in cages with raised screen bottoms. In the ad libitum feeding series they were kept in groups of four; in the equalized consumption series, where there were six rats in a group,

TABLE 1

History of preparation of soy bean oil meals[1]

Expeller meals

DEGREE OF HEAT TREATMENT	PRELIMINARY DRYING		CONVEYING		PREWARMING		EXPELLING THE OIL	
	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
	°C.	min.	°C.	min.	°C.	min.	°C.	min.
Low	100–112	8	82–90	7	90	13	Cold shaft 105	2.0
Medium	100–112	8	82–90	7	100–112	13	Hot shaft { 112–125	1.5
							130	1.0
High	100–112	8	82–90	7	100–112	13	Hot shaft { 140	1.5
							150	1.0

Hydraulic meals

DEGREE OF HEAT TREATMENT	PRELIMINARY DRYING		HEATING		COOKING		PRESSING	
	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
	°C.	min.	°C.	min.	°C.	min.	°C.	min.
Low	60–80	60	60	10	82	90	75–65	50–60
Medium	60–80	60	60	10	105	90	75–65	50–60
High	60–80	60	60	10	121	90	80–68	50–60

Solvent meal

PRELIMINARY HEATING		EXTRACTING	DRYING		AERATING AND COOLING	
Temperature	Time	Temperature	Temperature	Time	Temperature	Time
°C.	min.	°C.	°C.	min.	°C.	min.
60	10	45	98	15	78–45	10–20

[1] The expeller meals were prepared in the soy bean plant of Allied Mills, Inc., at Peoria, Illinois, with Anderson expellers. The hydraulic and solvent meals were prepared in the soy bean plants of the Archer-Daniels-Midland Company at Toledo, Ohio, and Chicago, Illinois, respectively.

each rat was kept in a separate cage. The animals were weighed at approximately the same time every 7 days. The length of the feeding period in all growth experiments was 56 days. For convenience these experiments have been grouped into three separate series, viz., 1, 2 and 3.

In series 1 the experiments determined the nutritive value of the protein of the raw soy beans and the various samples of commercial soy bean oil meal by the ad libitum type of feeding. Although most of the rations were given a protein content of 18 per cent, a few were formulated at 10 per cent. Two lots of rats were fed a diet containing casein—one lot at each protein level of 10 and 18 per cent.

As seen from the results, table 2, the ground soy beans supported only slight growth when fed at an 18 per cent protein level and no growth at 10 per cent. The soy bean oil meals also produced better growth with 18 per cent protein than at 10 per cent. The high temperature expeller meal excelled the low temperature expeller meal and the ground soy beans A by a considerable margin in both rate and efficiency of gains regardless of the level of protein. At the 18 per cent level, the high temperature expeller meal excelled the medium temperature expeller meal. The medium and high temperature hydraulic meals were about equal in nutritive value. These values, however, were significantly higher than for the protein of the low temperature hydraulic meal. The feeding of the commercial solvent soy bean oil meal resulted in good growth and a nutritive value of its protein equal to any of the other meals except the high temperature expeller meal. The nutritive value obtained for this solvent meal was more than three times that of the ground raw soy beans C. Casein was found to be superior to all samples of soy beans and soy bean oil meal in rate and efficiency of gains at a protein level of 10 per cent. At the 18 per cent level, however, casein was no better than the proteins of the high temperature soy bean oil meal prepared by the expeller process.

The average daily food intake in table 2 shows that the failure of the raw soy beans and low temperature meals to promote good growth was not due to a lack of food consumption. The similarity of food intake for the first few days suggests that the low nutritive value of the proteins of the raw soy beans and low temperature meals was due to some deficiency.

TABLE 2

The nutritive value of the proteins of the raw soy bean and soy bean oil meals prepared at different temperatures, compared with casein at protein levels of 10 and 18 per cent. Food intake ad libitum. (Series 1)[1]

LOT	DIET					LEVEL OF PROTEIN	AVERAGE DAILY FOOD INTAKE			GAIN IN BODY WEIGHT		GROWTH PER GRAM OF PROTEIN EATEN
	Casein	Soy beans			Supplement		3 days after 1st day	7 days after 1st day	Feeding period of 56 days	Range	Average	
		Kind	Process	Temperature								
	per cent					*per cent*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1		Gr. bean A		Raw		10	5.0	4.5	3.9	—12–+3	—(4.5)	
2		Oil meal	Expeller	105°C.		10	5.8	5.0	6.3	17– 39	23.5	0.65
3		Oil meal	Expeller	150°C.	Soy bean oil	10	6.8	6.8	7.2	20– 65	48.0	1.19
19	8.4				Agar (2 per cent)	10	6.3	6.5	8.8	26– 98	73.5	1.49
41		Gr. bean A		Raw		18	7.0	5.3	5.3	21– 36	27.3	0.51
6		Oil meal	Expeller	105°C.		18	4.5	5.0	5.7	18– 41	33.5	0.58
15		Oil meal	Expeller	130°C.	Soy bean oil	18	6.3	7.3	9.1	80–136	105.0	1.15
7		Oil meal	Expeller	150°C.	Soy bean oil	18	4.8	5.0	8.7	116–135	126.3	1.45
26		Oil meal	Hydraulic	82°C.	Soy bean oil	18	5.3	5.3	5.4	45– 78	59.7	0.82
17		Oil meal	Hydraulic	105°C.	Soy bean oil	18	6.0	7.0	10.3	77–190	120.3	1.14
18		Oil meal	Hydraulic	121°C.	Soy bean oil	18	7.3	7.5	10.5	103–134	122.3	1.15
45		Gr. bean C		Raw		18	4.5	4.8	5.0	11– 34	19.0	0.37
32		Oil meal	Solvent	98°C.		18	4.0	5.5	8.0	93–101	98.5	1.22
8	18.2				Agar (2 per cent)	18	5.3	6.3	10.4	141–163	150.8	1.44

[1] Each lot consisted of four rats.

In the experiments in series 2, table 3, the equalized type of feeding was employed. Six male rats of approximately the same age and weight constituted each experimental lot. All rats included in these experiments received the same total amount of food for the feeding period. This was determined by the intake of the rat which consumed the least food ad libitum, all lots considered. Adjustments were made in the daily portion of food weighed out so that the total food intake of each rat was the same by the end of each 7 days. The important advantage of this method over the paired feeding

TABLE 3

The nutritive value of the proteins of soy bean oil meals prepared at different temperatures. Equalized intake. (Series 2)[1]

LOT	DIET				GAIN IN BODY WEIGHT (56 DAYS)		GROWTH PER GRAM OF PROTEIN EATEN
	Soy beans			Supplement			
	Kind	Process	Temperature		Range	Average	
					gm.	gm.	gm.
33	Ground bean A		Raw		31–45	37.2	0.75
40	Oil meal	Expeller	105°C.		34–41	36.2	0.73
34	Oil meal	Expeller	150°C.	Soy bean oil	42–58	52.0	1.04
39	Ground bean B		Raw		35–46	39.0	0.78
36	Oil meal	Hydraulic	105°C.	Soy bean oil	45–60	53.0	1.06
35	Oil meal	Hydraulic	121°C.	Soy bean oil	46–63	54.8	1.10

[1] The level of protein for all diets was 18 per cent. The average daily food intake was 4.9 gm. Each lot consisted of six rats.

technic of Mitchell and Smuts ('32) is that it permits comparison of growth data obtained from one lot of animals whether treated individually or collectively with that of the animals of any lot within the same series. The constituents which formed the experimental diet for each lot in this series of experiments are given in table 3. The level of protein chosen for the diets was 18 per cent. The samples of ground raw soy beans A and B and the low temperature expeller soy bean oil meal were found to be equally inferior as a source of protein compared with the high temperature expeller meal and the medium and high temperature hydraulic meals.

In these growth experiments series 1 and 2 we found for the expeller and hydraulic soy bean oil meals a certain agreement between the color of the meals and the relative efficiency of the proteins. The inefficient low temperature expeller meal was light colored while the highly efficient medium and high temperature expeller meals were light brown and brown in color respectively. The less efficient low temperature hydraulic meal was also light colored while the more efficient medium and high temperature hydraulic meals were light brown and brown in color. In direct contrast with the expeller and hydraulic meals, the solvent meal, which contained an efficient protein, was light in color. These results suggest that brown color can only be used as an index of the probable efficiency of the proteins of commercial soy bean oil meals with the expeller and hydraulic meals.

The experiments of series 3 represent an initial attempt to determine the cause of the nutritive failure with raw soy beans. In some of our early experiments where the raw soy bean supplied the protein, excepting that contained in the yeast, the rats not only failed to make consistent growth but often showed a depraved appetite and unthrifty appearance usually leading to death in the sixth to the tenth week. The diets for each of the five lots in this series of experiments are listed with the results in table 4. The addition of casein to the ground soy bean diet resulted in good growth and a condition of well being. Due to the fact that the protein was fed at a high level its full nutritive value was not revealed. The results suggest that the cause for the failure of the ground raw soy bean to support appreciable growth in the rat in series 1 and 2 was directly due to a deficiency in good protein.

Removing the oil from the soy bean at room temperature did not result in any increase in the rate or efficiency of growth over that obtained in previous experiments where the ground raw soy beans were fed.

In lots where the ground whole soy bean, which had received various heat treatments, was included in the experimental diets, varying results were obtained. Dry heat for

1½ hours did not improve its nutritive value. This heated soy bean material had a color index similar to that of the high temperature expeller meal. Heating the ground soy bean in a sealed bomb for 1½ hours improved the nutritive value of the protein over that resulting from dry heating. In this process the original moisture of the ground bean was

TABLE 4

The growth promoting properties of soy beans as affected by additions of casein, by removal of the oil, and by various heat treatments. (Series 3)[1]

LOT	DIET			LEVEL OF PROTEIN	AVERAGE DAILY FOOD INTAKE			GAIN IN BODY WEIGHT		GROWTH PER GRAM OF PROTEIN EATEN
	Casein	Ground soy beans A								
		Process	Temperature		3 days after 1st day	7 days after 1st day	Feeding period of 56 days	Range	Average	
	per cent			*per cent*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
20	18		Raw	32	5.0	5.5	8.8	136–156	146.5	0.93
42		Heated in electric oven	135°C. 1½ hours	18	6.4	5.3	4.7	15– 30	22.3	0.48
50		Heated in sealed bomb in autoclave	17 lb. (125°C.) 1½ hours	18	6.3	6.8	8.9	45–116	83.3	0.93
51		Autoclaved	17 lb. (125°C.) 1½ hours	18	5.9	6.8	8.8	85–120	108.3	1.22
46		Oil extracted with ether	Room temperature 26°C.	18	6.2	6.6	5.5	9– 27	17.5	0.31

[1] Each lot consisted of four rats.

well preserved. The material was light brown in color. The most effective laboratory process for improving the nutritive value of the proteins of the ground whole soy bean proved to be that of autoclaving the ground material for 1½ hours with steam at 17 pounds pressure. This material was brown in color similar to that of the high temperature hydraulic soy bean oil meal.

Nitrogen balance experiments. These studies consisted of three nitrogen balance experiments designated respectively series 4, 5 and 6. Eight male rats of an average weight of about 85 gm. were used in each series. Series 4 and 5 consisted of two feeding periods each, while series 6 was made up of four periods, of which the first and fourth were used for feeding a nitrogen low ration. The periods were 10 days or more in length. The collection of excreta was made during the last 7 days of each period. The equalized intake type of feeding was employed in all. In each series the rats were divided into two lots of four rats each with the exception of the nitrogen free periods 1 and 4 of series 6. During the same period one lot was fed the raw soy bean diet and the other the heated soy beans. In the period following, the order of food assignment was reversed.

For the nitrogen balance experiments ground raw soy beans A and the high temperature expeller soy bean oil meal, processed at 140 to 150°C. for 2½ minutes, were fed in a basal ration in sufficient amounts to supply 18 per cent of protein. This basal ration was composed of about 45 parts cooked starch, 4 parts salts no. 40 (Steenbock and Nelson, '23) and 2 parts of cod liver oil. Soy bean oil was added in sufficient quantities to the diet containing soy bean oil meal to equal that supplied by the soy bean. The low nitrogen ration fed in periods 1 and 4, series 6, consisted of 15 parts butter fat, 8.4 parts soy bean oil, 2 parts cod liver oil, 4 parts salts no. 40, 4 parts agar, and 66.6 parts cooked starch. Vitamin B was supplied in the low nitrogen periods by giving each rat daily 40 mg. of an extract obtained from Parke, Davis and Company. This dosage of vitamin B extract contained 0.34 mg. of nitrogen.

The daily allowances of the experimental diets were weighed out on a sensitive balance. During the collection period the feces of each rat was transferred daily from the collector to small weighed flasks. These flasks contained a measured amount, usually 15 cc. of 2 per cent H_2SO_4. The urine collecting flasks contained about 10 cc. of a 2 per cent solution

of H_2SO_4. These flasks were emptied daily into large flasks which were of sufficient size to hold the 7-day collections plus washings. The surface of all glass-ware and other equipment exposed to the urine was washed daily with a warm 2 per cent solution of H_2SO_4 and distilled water. A double thickness of toweling was used to filter out any hairs or feed particles.

At the end of the collection period the feces were dried to constant weight at 100°C. The samples of feces were then ground to a uniform fineness in a steel mortar with a steel pestle and analyzed for nitrogen. The total urine collections were made up to a suitable volume from which an aliquot was analyzed for nitrogen.

The data for the low nitrogen periods are not presented in detail because the values obtained for the different rats were essentially similar. For example, the fecal nitrogen ranged from 5 to 7 mg. daily, the urinary nitrogen from 11 to 18 mg., the fecal nitrogen per gram of food eaten 1.4 to 1.9 mg., and the nitrogen in the urine per 100 gm. of body weight 16 to 20 mg. These values were for rats nos. 256 to 263, with the exception of rat 262 which was withdrawn from the experiment because it consumed some of its hair.

To conserve space the results of series 4 and 5 are not presented in detail. Briefly, in series 4 the raw and heated soy beans fed at 2.77 and 2.85 per cent of nitrogen gave apparent coefficients of digestibility of 77 and 85 per cent, respectively, with apparent biological values of 9 and 26. In this statement the adjective 'apparent' is used to designate the fact that the values have not been corrected for endogenous nitrogen according to the procedure of Mitchell and Villegas ('23) and Mitchell ('24). In series 5 at 2.85 and 2.95 per cent of nitrogen the respective values for heated and unheated beans were 80 and 83 for digestibility and 6 and 18 for apparent biological value.

The data of series 6 are presented in detail in table 5. However, only the corrected values are tabulated. It should be stated for purposes of comparison with series 4 and 5 that the apparent values with the raw and heated soy beans were,

TABLE 5

Nitrogen balances showing digestibility and biological values of raw and heated soy beans. (*Series 6*)

PERIOD	RAT NO.	BODY WEIGHT	DAILY FOOD INTAKE	DAILY FECAL N	DAILY URINE N	DAILY ENDOGENOUS N		DAILY FOOD N ABSORBED	DAILY FOOD N RETAINED	DIGESTION COEFFICIENT CORRECTED	BIOLOGICAL VALUE CORRECTED
						Fecal	Urine				
Raw soy bean ration 11 (2.89 per cent N)—daily N intake per rat = 145 mg.											
		gm.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
Second	256	82	5	31	90	10	16	124	50	85	40
Second	257	82	5	28	86	8	18	125	57	86	46
Second	258	85	5	34	78	7	15	118	55	81	47
Second	259	78	5	30	82	8	13	123	54	85	44
Third	260	92	5	30	99	10	16	125	42	86	34
Third	261	92	5	24	95	7	16	128	49	88	38
Third	263	96	5	30	88	8	15	123	50	85	41
									Average	85	41
Heated soy bean ration 13 (2.87 per cent N)—daily N intake per rat = 144 mg.											
Third	256	90	5	29	84	10	18	125	59	87	47
Third	257	90	5	30	82	8	19	122	59	85	48
Third	258	96	5	27	78	7	18	124	64	86	52
Third	259	90	5	25	72	8	15	127	70	88	55
Second	260	84	5	26	83	10	16	128	61	89	48
Second	261	84	5	27	76	7	15	124	63	86	51
Second	263	89	5	25	72	8	14	127	69	88	54
									Average	87	51

respectively, 80 and 81 for digestibility and 23 and 33 for biological value.

It is clearly shown that heating the raw soy bean caused an increase in the utilization of the absorbed nitrogen (biological value). The apparent biological values obtained in these experiments for the proteins of the soy bean are in agreement with the corrected values obtained in series 6. This harmony of results existing between uncorrected and corrected values for percentage of nitrogen retention is largely credited to both the short time of confinement for the rat of each series and the reversal plan of feeding.

The fact that heating increased the biological value of the protein helps to explain the increased gain per gram of protein which resulted from similar heat treatment in our growth experiments. While it is possible that the increase in efficiency of the protein resulted from the small increase in digestibility, it is not excluded that heat may have made available for metabolic use a protein fraction which was absorbed but not metabolically available. It remains to be seen if this can be established by metabolism trials for certain complexes as for example those which contain cystine. That cystine supplements raw soy bean proteins is well known (Mitchell and Smuts, '32; Shrewsbury and Bratzler, '33).

SUMMARY

Raw soy beans were found to contain protein of low nutritive value as determined by the grams of growth per gram of protein eaten. Commercial soy bean oil meals such as the expeller meal processed at low temperatures, 105°C. for 2 minutes or the hydraulic meal cooked at 82°C. for 90 minutes contained proteins similar in nutritive value to the raw soy beans. On the other hand, commercial soy bean oil meals which had been prepared at medium and high temperatures such as expeller meals processed at 112 to 130 and 140 to 150°C. for 2½ minutes or hydraulic meals cooked at 105 and 121°C. for 90 minutes contained proteins which had about twice the nutritive value of the raw soy beans or low temperature meals. These expeller and hydraulic meals prepared at

medium temperatures, respectively, were light brown in color while the meals prepared at high temperatures were brown in color. Heating the extracted soy beans at 98°C. for 15 minutes, as in the commercial solvent method of oil extraction, was also found to be an effective method of heat treatment. This solvent meal, however, was light colored. When the ground whole soy bean was autoclaved in the laboratory until the meal was brown in color, the protein had a high nutritive value. These results together with the fact that the commercial solvent meal was found to contain a very efficient protein suggest that brown color can only be used as an index of the probable efficiency of the proteins of commercial soy bean oil meals produced by the expeller and hydraulic processes.

The food intake of all rats which received either the raw or heated soy bean diets ad libitum was found to be similar for the first few days of the feeding period. This suggested that the poor growth resulting from the raw soy beans and low temperature meals was due to some deficiency in these constituents rather than to a lack of palatability.

When casein was incorporated in the diet which contained ground raw soy beans, normal growth resulted. These results suggested that the deficiency in the soy bean existed in the protein fraction.

Heating the raw soy bean to a high temperature in the expeller method of oil extraction caused an increase in the digestibility and biological value of the protein. The digestibility increased only about 3 per cent while the increase in biological value was about 12 per cent. This increase in biological or nutritive value of the protein established by metabolism tests agreed with the increase in nutritive value as determined by growth experiments.

The possibility appeared that heat caused some essential protein fraction, which was unavailable in the raw soy bean, to become available for absorption and metabolic use.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

VII. UTILIZATION OF INORGANIC ELEMENTS (A CONTINUOUS CASE STUDY OF A MULTIPARA)[1]

FRANCES COPE HUMMEL, HELEN R. STERNBERGER, HELEN A. HUNSCHER AND ICIE G. MACY

Research Laboratory, Children's Fund of Michigan, Detroit

THREE FIGURES

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As the studies on the metabolism of women during the reproductive cycle have progressed in this laboratory over a period of years, it has become increasingly apparent that—

it is expedient to extend, not only the short balance to a longer period of time, but to determine uninterruptedly the metabolic response of the same mother during the development of the fetus and the physiological preparation of her own body for lactation, and thereafter, to extend these observations into parturition, puerperium and lactation, thereby learning more specifically where the stresses and stains of child-bearing and child-rearing lay (Hunscher, Hummel, Erickson and Macy, '35).

Heretofore, nearly all the emphasis has been laid on a few of the more obvious inorganic elements in nutrition especially those that are known to be intimately related to protein, blood and bone formation to the exclusion of other similarly essential nutrients. Such substances may be needed in the body in only small quantities and even in traces, but nevertheless, they play important metabolic functions in the body

[1] The subject of this investigation has been referred to in other publications from this laboratory as L.R. and subject VII.

and may influence greatly through their chemical affinity or antagonism the utilizability of other substances in the body.

To date the quantitative studies[2] in pregnancy have included, for the most part, intermittent metabolic balances on calcium, magnesium, phosphorus with but one report each on iron, sulfur and acid-base mineral with the exception of Hoffström ('10) who observed continuously calcium, magnesium and phosphorus retentions and sulfur outgo on one woman from the seventeenth week of pregnancy to term. The only studies on inorganic balances in lactation and post lactation have been those reported by Toverud and Toverud ('31); Bauer, Albright and Aub ('29), and this laboratory (Hunscher, '30; Macy, Hunscher, McCosh and Nims, '30; Donelson, Nims, Hunscher and Macy, '31). Heretofore, no studies have been made continuously during the entire reproductive cycle including pregnancy, puerperium and lactation. The present paper records data pertaining to the progressive utilization or loss and the continuous chemical exchange of calcium, magnesium, sodium, potassium, sulfur, phosphorus and chlorine by a multipara during 21 weeks of pregnancy, and after 10 days' puerperium to the eighth week postpartum, an observation period approximating 8 months; for comparative purposes data collected from balances made at intervals during the two preceding reproductive cycles are included.

EXPERIMENTAL

Subject. An American woman (L.R.)[1] who had participated over a 5-year period in many types of nutritional and physiological studies relating to the metabolism of the reproductive cycle, including the chemistry of milk and factors affecting milk secretion, offered her services again for this long and intensive metabolic study during the early part of her fourth reproductive cycle, of which the data are recorded herein and in a previous publication (Hunscher, Hummel, Erickson and Macy, '35). Frequent medical examinations

[2] An inclusive bibliography on mineral utilization in pregnancy has not been compiled since several comprehensive reviews have appeared recently.

not only during the second and third reproductive cycles but during the present or fourth verified the absence of metabolic disturbances, and the usual dyscrasias of conception; for instance, there was no nausea, no gastro-intestinal disturbances, no albuminuria, no unusual changes in body weight during either pregnancy or lactation.[3] While under the observations of the staff of this laboratory during her second and third reproductive cycles she had successfully borne nutritionally stable children who have continued to develop both physically and mentally in a most satisfying manner without known metabolic disturbances as well as few infections and diseases of childhood. During her three initial lactation periods and the present one she secreted considerable quantities of breast milk beyond what her babies needed because she gave herself a maximum stimulus of complete and frequent removal of all the milk from the mammae (Macy, Hunscher, Donelson and Nims, '30). The quantity of milk was no greater perhaps than the average mother would produce who successfully bears and breast feeds twins nor any other healthy robust woman who has the inherent ability to secrete milk were the same stimulus persistently applied and were analagous hygiene and sanity of living rigidly practiced. After many years of careful study of the physiological functions of child-bearing and child-rearing we have grown to consider this woman approaching the optimal because she has so successfully carried out all the functions of motherhood in an average sized family and, in addition, maintained her own self in an admittedly superior state of health. Certainly with our present medical and nutritional knowledge of the physiology of maternity it would be difficult to offer any specific suggestions for improvement of either the physical well-being of this woman or her offspring.

Dietary. The diet was voluntarily chosen as to quantity and quality and it included 2 quarts of milk in each of which were incorporated 400 U.S.P. units of cod liver oil concentrate

[3] The authors are grateful to A. S. Guimaraes, M.D., for his interest and cooperation.

(Vitex).[4] The diet of her own free choice was abundant in all known nutritive essentials and in this food consumption there was an average of 3389 (2490 to 4324) calories and of 19 (16.8 to 22.33) gm. of nitrogen (Hunscher, Hummel, Erickson and Macy, '35).

General procedure. The subject remained in her own home performing her usual home duties except during delivery and the lying-in period.[5] Successive metabolic mineral balances of 5 days' duration were begun in the fourth reproductive cycle at approximately the one hundred and thirty-fifth day after conception and carried thereafter continuously through the first 2 months of lactation with only an interruption of the initial 10 days' postpartum. The metabolic collection procedures adhered to in this laboratory and described in a previous publication (Hunscher, Hummel, Erickson and Macy, '35) were followed.

Methods. The inorganic elements in the urine were determined according to the following procedures. Calcium and magnesium were determined by the McCrudden method ('09–'10; '11) with certain adaptations. The precipitated calcium oxalate was finally washed with cold water, dissolved in hot H_2SO_4 and titrated with 0.05 N potassium permanganate. On two occasions, however, when large amounts of urates were present in the urine the use of the method of Shohl and Pedley ('22) seemed advantageous. In all cases the filtrate and washings were used for the determination of magnesium as outlined in the McCrudden procedure. For the sodium and potassium analyses the combined sulfates were obtained and weighed according to the classic Lindo-Gladding ('24) method of the Association of Official Agricultural Chemists as adapted to biological materials by Mackay and Butler and given in Peters and Van Slyke ('32). Sodium was determined on a

[4] We wish to express our thanks and appreciation to Bion East, D.D.S., of the Vitex Laboratories, Inc., Harrison, N. J., for furnishing the fortified vitamin D milk for this study.

[5] It is a pleasure to acknowledge the helpful cooperation of the medical, nursing and dietetic staffs of the Florence Crittenton Hospital, Detroit, in the care of the patient during the 10 days' confinement period.

fresh sample of urine by the uranyl acetate micro gravimetric method of Barber and Kolthoff as modified by Butler and Tuthill ('31). The amount of sodium sulfate was then calculated and the potassium obtained by difference. The other inorganic elements were determined by the following procedures: chlorine by the Volhard-Arnold titration method described in Hawk and Bergeim, '31; phosphorus by the colorimetric micro method of Youngburg and Youngburg, '30; and sulfur by the Benedict ('09) gravimetric method for total sulfur in urine.

In the case of food and feces the above methods were used for sodium, chlorine, phosphorus and sulfur but for the other elements certain changes seemed advantageous in our hands. In determining calcium the micro titration method by Hawks ('31) was used with samples of 2.0 to 2.5 gm. of food and approximately 1.75 gm. of feces. For magnesium, the Greenberg and Mackey method ('32) was adapted with the following modifications: the supernatant fluid and washings from the calcium precipitation were evaporated to dryness on the hot plate with 5 cc. of HNO_3. The residue was dissolved in 25 cc. of water to which 1 cc. of 2 per cent NH_4Cl, 2 cc. of 2 per cent 8-hydroxyquinoline (in 95 per cent alcohol), 20 drops of NH_4OH were added and the whole thoroughly mixed. The solution was then heated on a steam bath for 25 minutes, transferred to filter with three minimal portions of 2 per cent NH_4OH, and finally washed six times with water. The precipitate was dissolved in 20 cc. hot 1:4 HCl and the filter washed six times with water. One cubic centimeter of saturated KBr and 5 cc. of M/30 $KBrO_3$ were added, the tube was then stoppered, shaken and let stand for 30 to 60 seconds. One cubic centimeter of 20 per cent KI was added and the solution titrated with 0.05 N $Na_2S_2O_3$ to a starch endpoint. The cobaltinitrite micro titration method of Clausen ('18), and Kramer and Tisdall ('21), as outlined by Peters and Van Slyke ('32) was used for potassium.

RESULTS AND DISCUSSION

Tables 1 to 3 give a summary of the metabolic data accumulated on the continuous balances of the base-forming inorganic elements, calcium, magnesium, sodium, potassium and the acid-forming inorganic elements of phosphorus, sulfur and chlorine of one woman (L.R.) in her fourth reproductive cycle. The quantities of these inorganic elements taken into the body over a period of approximately 8 months beginning with the final 145 days of gestation and from the tenth to fifty-third days postpartum including the period of heaviest milk flow (Macy, Hunscher, Donelson and Nims, '30), the paths of excretion of the same elements in the urine, feces and breast milk, together with maternal gain or loss are recorded. In addition, the present data are enhanced in value through their supplementation by results from individual short time calcium balance periods at intervals in the second lactation and during the third cycle including post lactation as shown in figure 1 (Hunscher, '30; Macy, Hunscher, McCosh and Nims, '30; Donelson, Nims, Hunscher and Macy, '31).

Pregnancy. The results of the calcium, magnesium, sodium and potassium balances are given in table 1 and of phosphorus, sulfur and chlorine in table 2. For the most part in gestation positive retentions predominated in the case of all the observed inorganic elements but there were noticeable fluctuations from period to period and even losses in some instances. With the mean daily intake of 3.09 ± 0.15, 0.60 ± 0.02, 5.05 ± 0.51 and 6.60 ± 0.56 gm. of calcium, magnesium, sodium and potassium, the mean maternal retentions were 0.37 ± 0.19, 0.11 ± 0.05, 0.56 ± 0.53 and 1.40 ± 0.72 gm., respectively, and mean intake of 2.67 ± 0.22, 1.50 ± 0.15 and 7.68 ± 0.96 of phosphorus, sulfur and chlorine the mean retentions were 0.26 ± 0.16, 0.34 ± 0.13 and 0.60 ± 0.96 gm., respectively. From these data it will be noted from table 4 that the mean daily balances for calcium, magnesium, potassium and sulfur were greater in the present uninterrupted study in pregnancy on one woman than those obtained in previous observations in which isolated periodic balances

TABLE 1

Inorganic acid-forming elements retained per day by a woman (L.R.) who was studied uninterruptedly throughout the last half of pregnancy in her fourth reproductive cycle

DAY OF GESTATION	CALCIUM				MAGNESIUM				SODIUM				POTASSIUM			
	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
135–141	2.86	0.43	2.18	0.25	0.56	0.15	0.38	0.03	4.99	4.35	0.05	0.59	6.07	3.48	0.73	1.86
142–148	3.05	0.45	2.44	0.16	0.66	0.17	0.44	0.05	5.43	4.62	0.05	0.76	6.38	3.54	0.79	2.05
149–155	3.07	0.44	2.28	0.35	0.53	0.17	0.33	0.03	4.92	4.27	0.08	0.57	5.88	3.76	0.69	1.43
156–162	2.96	0.42	2.28	0.26	0.64	0.16	0.41	0.07	4.26	4.16	0.06	0.04	7.14	4.76	0.82	1.56
163–167	2.94	0.44	2.22	0.28	0.58	0.16	0.39	0.03	5.46	4.52	0.09	0.85	6.88	4.70	0.67	1.51
168–170	2.90	0.36	2.02	0.52	0.59	0.11	0.42	0.06	4.36	3.31	0.09	0.96	6.72	4.65	0.95	1.12
171–175	3.11	0.36	2.32	0.43	0.71	0.14	0.43	0.14	5.34	3.44	0.07	1.83	7.58	4.90	0.87	1.81
176–180	3.15	0.42	2.15	0.58	0.56	0.07	0.33	0.16	6.01	5.75	0.08	0.18	6.44	4.66	0.59	1.19
181–185	3.06	0.50	2.25	0.31	0.61	0.16	0.39	0.06	5.28	4.96	0.13	0.19	6.33	4.21	0.68	1.44
186–190	2.89	0.38	1.97	0.54	0.48	0.08	0.30	0.10	2.87	2.99	0.21	—0.33	5.38	3.67	0.73	0.98
191–195	3.17	0.33	2.48	0.36	0.76	0.10	0.45	0.21	5.13	3.78	0.09	1.26	7.26	3.25	0.86	3.15
196–200	3.08	0.33	2.09	0.66	0.61	0.10	0.37	0.14	4.66	3.62	0.09	0.95	6.56	5.24	0.69	0.63
201–205	2.78	0.38	2.11	0.29	0.50	0.11	0.29	0.10	4.11	4.06	0.10	—0.05	5.77	4.71	0.59	0.47
206–210	3.04	0.41	2.44	0.19	0.65	0.11	0.43	0.11	5.57	4.95	0.11	0.51	7.25	3.60	0.69	2.96
211–215	3.18	0.42	2.50	0.26	0.55	0.11	0.36	0.08	5.18	4.97	0.12	0.08	7.05	4.87	0.71	1.47
216–220	3.00	0.39	2.28	0.33	0.52	0.14	0.31	0.07	5.59	4.55	0.08	0.96	6.42	4.30	0.63	1.49
221–225	3.17	0.43	2.65	0.09	0.66	0.11	0.45	0.10	6.24	4.96	0.09	1.19	7.11	4.51	0.70	1.90
226–230	3.35	0.40	2.35	0.60	0.62	0.12	0.52	0.10	5.13	4.94	0.17	0.02	7.49	5.21	0.71	1.57
231–235	3.08	0.46	2.30	0.32	0.58	0.13	0.32	0.13	4.51	4.37	0.08	0.06	6.91	5.09	0.57	0.25
236–240	3.16	0.44	2.33	0.39	0.57	0.09	0.34	0.14	5.14	5.09	0.13	—0.08	6.77	5.01	0.73	1.03
241–245	2.97	0.44	2.36	0.17	0.62	0.15	0.38	0.09	5.28	4.95	0.08	0.25	7.19	5.36	0.59	1.24
246–250	3.14	0.49	2.50	0.15	0.72	0.11	0.38	0.23	5.21	3.83	0.10	1.28	7.25	5.35	0.79	1.11
251–255	3.28	0.41	2.36	0.51	0.57	0.17	0.31	0.09	4.41	3.07	0.11	1.23	5.93	5.76	0.53	—0.36
256–260	3.11	0.51	1.99	0.61	0.58	0.16	0.31	0.11	5.03	4.54	0.07	0.42	6.01	3.13	0.58	2.30
261–265	3.25	0.50	2.22	0.53	0.59	0.10	0.32	0.17	4.97	4.46	0.08	0.43	6.11	4.42	0.59	1.10
266–270	3.16	0.39	2.58	0.19	0.60	0.11	0.40	0.09	4.45	3.24	0.07	1.14	6.14	3.98	0.52	1.64
271–275	3.33	0.43	1.99	0.91	0.62	0.13	0.29	0.20	4.31	3.62	0.03	0.66	6.26	4.49	0.40	1.37
276–280	3.29	0.46	2.69	*0.14*	0.62	0.17	0.39	*0.06*	4.99	4.88	0.26	*—0.15*	6.57	4.91	0.81	*0.85*
Total gain—145 days (final)				*52.90*				*15.48*				*81.00*				*207.16*
Mean	*3.09*			*0.37*	*0.60*			*0.11*	*5.05*			*0.56*	*6.60*			*1.40*

TABLE 2

Inorganic acid-forming elements retained per day by a women (L.R.) who was studied uninterruptedly throughout the last half of pregnancy in her fourth reproductive cycle

DAY OF GESTATION	PHOSPHORUS				SULFUR				CHLORINE			
	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
135–141	2.64	1.36	1.08	0.20	1.62	0.96	0.17	0.49	7.32	6.73	0.09	0.50
142–148	2.85	1.29	1.23	0.33	1.57	1.05	0.23	0.29	8.05	6.68	0.10	1.27
149–155	2.68	1.51	1.06	0.11	1.38	0.97	0.18	0.23	7.56	6.42	0.12	1.03
156–162	2.67	1.20	1.17	0.30	1.72	0.96	0.18	0.58	7.86	7.05	0.11	0.70
163–167	2.70	1.26	1.16	0.28	1.51	1.01	0.18	0.32	8.42	7.47	0.11	0.84
168–170	2.49	1.00	1.13	0.36	1.39	0.97	0.16	0.26	5.95	6.08	0.09	—0.18
171–175	3.08	1.29	1.13	0.66	1.86	1.07	0.23	0.56	7.93	5.75	0.11	2.07
176–180	2.58	1.25	0.91	0.42	1.43	0.96	0.15	0.32	9.17	9.12	0.08	—0.03
181–185	2.61	1.61	0.95	0.05	1.55	1.13	0.20	0.22	7.81	7.71	0.12	—0.02
186–190	2.24	1.07	0.88	0.29	1.13	0.82	0.15	0.16	4.65	7.08	0.10	—2.53
191–195	3.08	1.39	1.34	0.35	1.69	0.95	0.23	0.51	7.49	5.72	0.09	1.68
196–200	2.73	1.14	1.03	0.56	1.47	0.92	0.16	0.39	7.06	5.59	0.09	1.38
201–205	2.41	1.19	0.97	0.25	1.40	0.84	0.19	0.37	6.42	6.30	0.10	0.02
206–210	2.85	1.47	1.14	0.24	1.52	1.01	0.19	0.32	8.49	7.50	0.11	0.88
211–215	2.77	1.59	1.10	0.08	1.71	1.03	0.19	0.49	8.28	8.11	0.12	0.05
216–220	2.67	1.56	1.06	0.05	1.49	1.10	0.15	0.24	8.55	6.99	0.09	1.47
221–225	2.81	1.54	1.15	0.12	1.58	1.04	0.18	0.36	9.35	7.54	0.09	1.72
226–230	2.72	1.32	0.91	0.49	1.53	1.02	0.17	0.34	8.16	7.62	0.14	0.40
231–235	2.69	1.54	0.85	0.30	1.62	0.99	0.14	0.49	7.26	7.16	0.09	0.01
236–240	2.61	1.40	0.98	0.23	1.44	1.06	0.16	0.22	8.31	8.09	0.15	0.07
241–245	2.61	1.41	0.98	0.22	1.71	0.98	0.18	0.55	8.50	7.89	0.08	0.52
246–250	2.76	1.56	1.08	0.12	1.45	0.98	0.20	0.27	8.44	6.27	0.11	2.06
251–255	2.51	1.52	0.90	0.09	1.23	0.96	0.15	0.12	6.97	5.10	0.10	1.77
256–260	2.68	1.49	0.99	0.20	1.47	1.00	0.16	0.31	7.82	7.59	0.08	0.15
261–265	2.66	1.69	0.82	0.15	1.40	0.90	0.17	0.33	7.67	7.16	0.11	0.40
266–270	2.43	1.52	0.96	—0.05	1.28	0.84	0.19	0.25	7.07	5.81	0.09	1.17
271–275	2.70	1.50	0.70	0.50	1.48	0.98	0.13	0.37	6.62	6.33	0.06	0.22
276–280	2.71	1.40	1.02	*0.29*	1.47	0.94	0.23	*0.30*	7.73	8.41	0.22	—*0.90*
Total gain—final 145 days				*37.11*				*50.96*				*91.26*
Mean	*2.67*			*0.26*	*1.50*			*0.34*	*7.68*			*0.60*

TABLE 3

Inorganic base-forming and acid-forming elements retained per day by a woman (L.R.) who was studied uninterruptedly from the tenth to fifth-third day of lactation in her fourth reproductive cycle

DAYS POST-PARTUM	INTAKE	URINE	FECES	MILK	BALANCE	INTAKE	URINE	FECES	MILK	BALANCE
	Calcium					Magnesium				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
10–17	3.00	0.45	2.63	0.84	—0.92	0.56	0.10	0.42	0.15	—0.11
18–22	3.24	0.19	2.51	0.53	+0.01	0.63	0.04	0.41	0.12	+0.06
23–27	3.15	0.28	3.93	0.61	—1.67	0.65	0.07	0.87	0.11	—0.40
28–32	3.22	0.12	3.00	0.60	—0.50	0.63	0.10	0.42	0.11	0.00
33–37	3.09	0.06	2.23	0.65	+0.15	0.58	0.11	0.35	0.12	0.00
38–42	3.04	0.07	2.43	0.61	—0.07	0.71	0.05	0.44	0.12	+0.10
43–47	3.53	0.06	3.00	0.72	—0.25	0.72	0.10	0.51	0.12	—0.01
48–53	3.19	0.07	3.18	0.52	—0.58	0.86	0.15	0.53	0.09	+0.09
Total for 43 days of lactation					*—20.99*					*—1.57*
Mean	*3.18*				*—0.48*	*0.67*				*—0.03*
	Sodium					Potassium				
10–17	4.80	2.63	0.15	0.46	+1.56	6.19	3.96	0.71	1.43	+0.09
18–22	4.69	3.73	0.11	0.42	+0.43	6.33	3.93	0.61	1.10	+0.69
23–27	5.64	4.75	0.31	0.36	+0.22	7.20	4.51	1.12	1.06	+0.51
28–32	5.74	4.81	0.14	0.28	+0.51	7.34	4.76	0.68	1.52	+0.38
33–37	4.69	4.10	0.08	0.32	+0.19	5.83	4.40	0.51	1.19	—0.27
38–42	5.86	5.12	0.09	0.27	+0.38	7.00	1.84	0.57	1.18	+3.41
43–47	5.82	4.79	0.10	0.31	+0.62	7.57	4.31	0.69	1.32	+1.25
48–53	5.21	4.89	0.17	0.23	—0.08	8.31	5.69	0.74	1.01	+0.87
Total for 43 days of lactation					*+22.27*					*+34.83*
Mean	*5.31*				*+0.47*	*6.97*				*+0.87*
	Phosphorus					Sulfur				
10–17	2.59	1.58	1.14	0.42	—0.55	1.30	0.99	0.18	0.05	+0.08
18–22	2.63	1.49	1.10	0.25	—0.21	1.20	0.80	0.17	0.48	—0.25
23–27	2.87	1.40	1.90	0.28	—0.71	1.28	1.03	0.35	0.53	—0.63
28–32	2.76	1.28	1.19	0.28	+0.01	1.25	0.75	0.20	0.45	—0.15
33–37	2.61	1.83	0.97	0.33	—0.52	1.29	0.82	0.14	0.54	—0.21
38–42	2.91	1.73	1.10	0.35	—0.27	1.55	0.89	0.18	0.67	—0.19
43–47	2.96	1.91	1.24	0.40	—0.59	1.59	0.92	0.20	0.81	—0.24
48–53	3.13	1.64	1.30	0.30	—0.10	1.67	1.04	0.21	0.57	—0.15
Total for 43 days of lactation					*—15.80*					*—8.70*
Mean	*2.81*				*—0.37*	*1.39*				*—0.22*
	Chlorine									
10–17	7.83	4.51	0.20	0.87	+2.25					
18–22	7.19	5.65	0.13	0.87	+0.54					
23–27	8.66	7.70	0.22	0.81	—0.07					
28–32	9.22	7.70	0.11	0.76	+0.65					
33–37	7.39	6.61	0.09	0.95	—0.26					
38–42	8.63	7.54	0.10	0.75	+0.24					
43–47	8.65	7.58	0.09	0.95	+0.03					
48–53	7.47	7.55	0.18	0.71	—0.97					
Total for 43 days of lactation					*+16.55*					
Mean	*8.13*				*+0.39*					

TABLE 4

Summary of inorganic base-forming and acid-forming elements retained per day by a multipara (L.R.) during pregnancy and lactation compared with data from the literature

	PREGNANCY								LACTATION			
	Intake				Balance				Intake, present study		Balance, present study	
	Present study		Macy-Hunscher,[1] mean	Coons et al.,[2] mean	Present study		Macy-Hunscher,[1] mean	Coons et al.,[2] mean				
	Mean	Standard deviation			Mean	Standard deviation			Mean	Standard deviation	Mean	Standard deviation
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Calcium	3.09	0.15	1.44	1.45	0.37	0.19	0.17	0.28	3.18	0.15	—0.48	0.56
Magnesium	0.60	0.02	0.34	0.39	0.11	0.05	0.06	0.06	0.67	0.03	—0.03	0.15
Sodium	5.05	0.51		4.91	0.56	0.53		1.26	5.31	0.49	+0.47	0.46
Potassium	6.60	0.56		3.64	1.40	0.72		0.51	6.97	0.80	+0.87	1.06
Phosphorus	2.67	0.22	1.91	1.64	0.26	0.16	0.31	0.30	2.81	0.18	—0.37	0.24
Sulfur	1.50	0.15		0.76	0.34	0.13	0.05	0.004	1.39	0.17	—0.22	0.18
Chlorine	7.68	0.96		5.82	0.60	0.96		0.89	8.13	0.70	+0.39	0.88
Base (excess)[3]	1221	164		1037	347	246		436	1350	292	471	423

[1] Macy, I. G., and H. A. Hunscher, '34.

[2] Coons, Coons and Schiefelbusch, '34.

[3] Amount of base in excess of acid (0.1 N).

were the source of information on these minerals (Macy and Hunscher, '34; Coons, Coons and Schiefelbusch, '34). From the continuous metabolic balances in the present study there

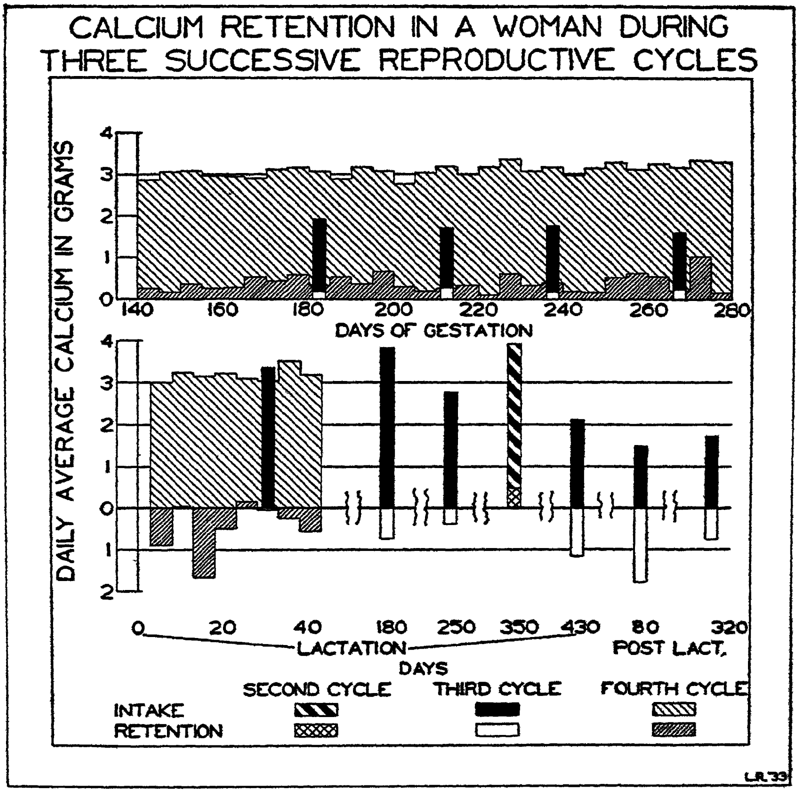

Fig. 1 Shows the continuous large calcium storage by a multipara during the last 145 days of pregnancy and from the tenth to fifty-third day of lactation in her fourth reproductive cycle when she was taking a diet fortified with a generous supply of minerals and vitamin D. Superimposed upon this curve are isolated 5- and 10-day balances that were made during two preceding cycles.

was no relationship of the amount of the elements retained to the progressive needs of gestation nor to the fluctuations of the intake. In the third reproductive cycle of the present subject at the twenty-sixth, thirtieth, thirty-fourth and thirty-

eighth weeks 4-day metabolic balances were conducted. The average daily calcium intakes were 1.92, 1.71, 1.76 and 1.60 gm. and the resultant retentions were 0.185, 0.278, 0.163 and 0.234 gm., respectively, and the average daily phosphorus intakes were 2.75, 2.27, 2.16 and 2.04 gm. which were accompanied by retentions of 0.712, 0.064, 0.135 and 0.468 gm., respectively (fig. 1).

If calcium, magnesium, sodium and potassium are considered totally responsible for the base, and chlorine, phosphorus and sulfur for the acid in the calculation of the acid-base mineral balances, the intake during pregnancy amounted to a mean of 1221 $\pm$ 164 cc. 0.1 N excess base per day accompanied by a mean retention of 347 $\pm$ 246 cc. (table 4). This is a greater daily storage of base than was calculated by Shohl ('23) when he used the composition of the fetus only as the basis of estimating maternal needs. From the average daily deposit of 85 cc. 0.1 N base in excess of acid in the fetus during the last 100 days of pregnancy he estimated that the needs of the gravid woman would be met by 150 cc. 0.1 N base per day. According to the latest figures available on the acid-base mineral content of the fetus (Givens and Macy, '33; Iob and Swanson, '34) a small proportion of the total retention of the mother was utilized in fetal development in the present subject, that is, 6.8, 2.3, 46.0 and 5.2 per cent of sodium, potassium, calcium and magnesium, respectively, and 1.6, 40.3 and 5.8 for sulfur, phosphorus and chlorine, respectively, were stored by the fetus. The greatest proportional deposit in the fetus at term in respect to the total amount retained during the latter half of pregnancy was in calcium and phosphorus; these elements were also shown to predominate in fetal chemical composition (fig. 2). Our results on acid-base mineral retention are comparable to those of Coons and co-workers (Coons, Coons and Schiefelbusch, '34; Coons, Schiefelbusch, Marshall and Coons, '35) who have recently reported a study on the acid-base mineral metabolism on five women at intervals during gestation (twenty acid-base balances) in which they showed an average storage of 436 $\pm$ 28 cc. 0.1 N base

in excess of acid daily. Only one of their twenty balances resulted in a retention less than Shohl's calculated requirement.

The uninterrupted balances in pregnancy show the total maternal accumulation of the individual elements which were used in fetal growth and maternal reserve in preparation for motherhood. In addition, on the one hand, they permit a study of the comparative rates of gains of the elements and their relationships to one another, and on the other hand, the detection of rates of changes in progressive gestatory needs. In contrast the short time metabolic studies, at intervals during the advancing reproductive cycle, give information upon food utilization at a specific time and under the existing physiological activities but because of the wide fluctuations of retentions even on a constant dietary intake, too rigid interpretations may lead to erroneous conclusions. During the final 145 days of pregnancy L.R. stored a total of 52.90, 15.48, 81.00 and 207.16 gm. of calcium, magnesium, sodium and potassium, respectively, and at the same time 37.11, 50.96 and 91.26 gm. of phosphorus, sulfur and chlorine, respectively, all of which constitute both fetal and maternal enhancement. Unfortunately, the metabolic balance method of study does not permit an exact differentiation of fetal and maternal utilization (Hunscher, Hummel, Erickson and Macy, '35). Rough approximations, however, can be made by calculating progressive fetal needs from the chemical composition of specimens obtained at autopsy; because of the nature of the material these data may or may not represent the normal mineral utilization in utero by the fetus. For example, by application of this method of study, of the 52.90 gm. of calcium laid down by L.R. from about the one hundred and thirty-fifth day to term, approximately 24 gm. went to the fetus leaving a known maternal reserve of about 29 gm. (fig. 2). Since this mother did not experience any of the usual dyscrasias following conception it is within reason to assume that the final maternal storage of pregnancy was even greater than recorded in this report since she, no doubt, was acquiring

considerable quantities of the various nutrients during the initial half of the period before these quantitative observations were begun. Similarly, the fetal requirements and the maternal reserves for the other six elements can be computed. The total storage of calcium, phosphorus and magnesium in our subject are comparable with those recorded by Hoffström ('10), the only other uninterrupted study that has been made in the last half of the gravid period.

The rates of gain of the seven inorganic elements and nitrogen in pregnancy are shown in figures 2 and 3. These curves were plotted on semi-logarithmic paper so as to illustrate the relative rates of gain of the different elements with the progression of the reproductive cycle and, in addition, shows their actual total accumulation during the final 145 days of gestation. It is of interest to note that the rate of accumulation of nitrogen, sulfur, potassium and sodium are similar; with the exception of sodium these elements predominate in the formation of soft tissue. Chlorine, after the one hundred and eightieth day in the period of pregnancy approximates the rate of gain in calcium and phosphorus, all of which are more rapidly accumulated than the above elements. Calcium and phosphorus are notable constituents in bone formation. In contrast, after the one hundred eightieth day the rate of magnesium storage was more rapid than the other seven elements which may be an assalient feature when considered in the light of the magnesium content of human embryos (Givens and Macy, '33). Together with supplemental studies[6, 7] of various types in progress in this laboratory these relationships will be given further detailed considerations. It can be stated, however, from data presented in the semi-logarithmic charts that in the last half of pregnancy there was no apparent change in the progressive gestatory needs as might have been inferred had isolated balances per-

[6] Continuous inorganic mineral balances on a young primipara is to be published shortly.

[7] An extensive study on the successive metabolic balances in childhood will be published in the near future.

chance been made at intervals when physiological and psychological conditions were such that made possible greater storage of the various nutrients and therefore, larger metabolic balances (fig. 1).

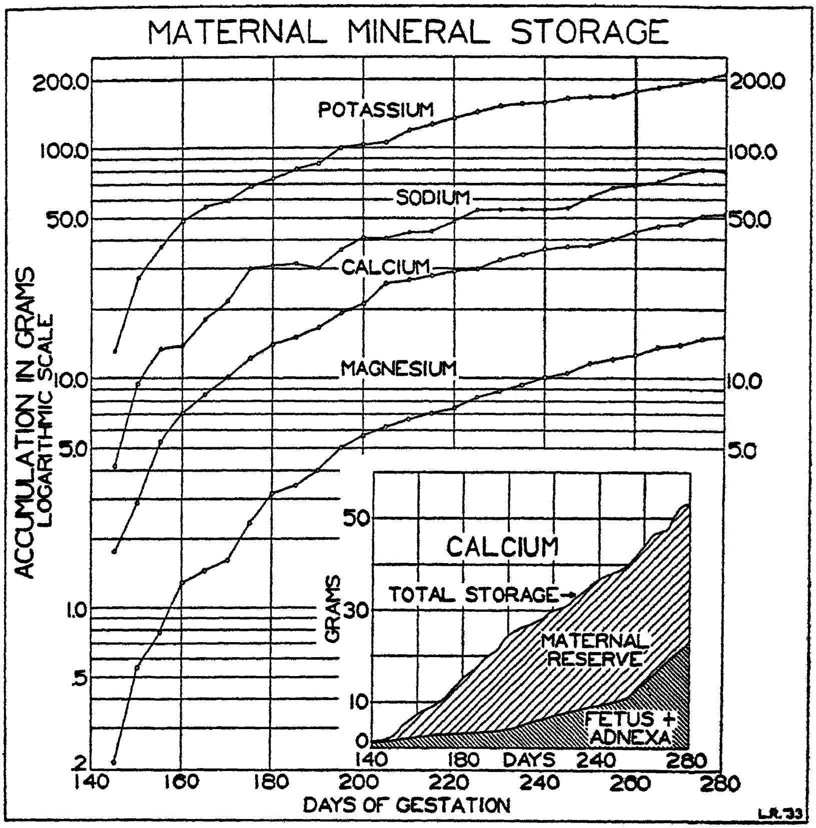

Fig. 2 Shows the continuous maternal storage of potassium, sodium, calcium and magnesium during the final 145 days of gestation. The days are plotted arithmetically and the maternal accumulation in grams logarithmically thus illustrating not only the absolute values but the relative rates of change with the progression of fetal development. The insert shows the large determined maternal reserve of calcium that is stored beyond the estimated needs of the fetus and its adnexa.

Lactation. In contrast to pregnancy, the physiological activity of lactation resulted in lowered retention and, in some cases, negative balances. Tables 3 and 4 show the data on

the continuous metabolic balances from the tenth to fifty-third day postpartum. The mean daily intakes and their standard deviations of the base-forming inorganic elements calcium, magnesium, sodium and potassium were 3.18 ± 0.15, 0.67 ±

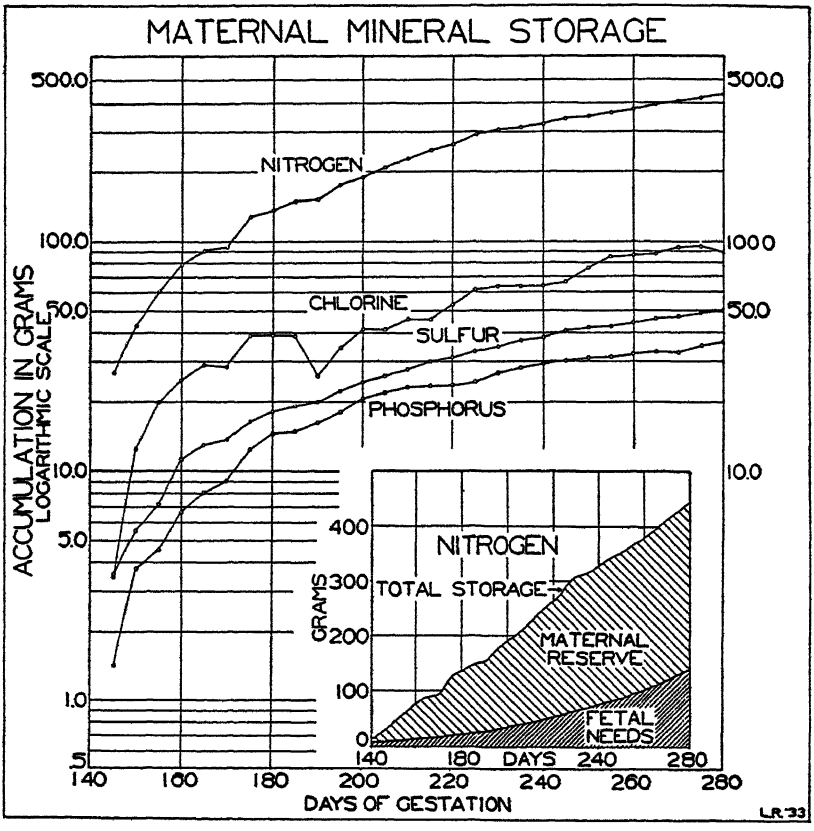

Fig. 3 Shows the continuous storage of nitrogen, chlorine, sulfur and phosphorus during the final 145 days of gestation. The days are plotted arithmetically and the maternal accumulation in grams logarithmically thus illustrating the absolute values and the relative rates of change with the progression of fetal development. The insert shows the large determined maternal storage of nitrogen that has been laid down by the mother in excess of the fetal needs.

0.03, 5.31 ± 0.49 and 6.97 ± 0.80 gm. which resulted in metabolic balances of — 0.48 ± 0.56, — 0.03 ± 0.15, + 0.47 ± 0.46, + 0.87 ± 1.06 gm., respectively, while the mean daily intakes

were 8.13 ± 0.70, 2.81 ± 0.18, 1.39 ± 0.17 gm. for the acid-forming chlorine, phosphorus and sulfur which were followed by balances of 0.39 ± 0.88, — 0.37 ± 0.24, — 0.22 ± 0.18 gm., respectively.

We have been unusually fortunate in securing data at intervals on the present subject in lactation during her second and third reproductive cycles. In the former cycle in the fiftieth week of lactation after a long and heavy milk flow when her intake of calcium was 3.92 gm. she was storing an average of 0.486 gm. per day but during the third cycle at the seventh, twenty-sixth, thirty-sixth and sixty-second weeks of lactation the intakes of calcium were 3.36, 3.83, 2.76 and 2.11 with the resultant balances of — 0.06, — 0.75, — 0.38 and — 1.16 gm. It is significant to note that the loss of calcium was diminished during the thirty-sixth week when a daily administration of 15 gm. of cod liver oil and 10 gm. of yeast had been given for the preceding 2 months (Donelson, Nims, Hunscher and Macy, '31).

The acid-base mineral balances of lactation in the present study when calculated by the usual method showed a mean intake of 1350 ± 292 cc. 0.1 N excess base followed by a mean retention of 471 ± 423 cc. 0.1 N excess base per day (table 4). To our knowledge these data represent the initial acid-base mineral balances in human lactation.

One of the most interesting observations in this continued metabolic study occurs in the chemical exchange in pregnancy as contrasted with lactation when there is a decrease from about 0.400 gm. of calcium in the urine daily during the intra-uterine development to about 0.065 gm. after the thirty-third day of lactation. These findings corroborate previous observations on the same subject and on two other women in early lactation (Donelson, Nims, Hunscher and Macy, '31).

It has been demonstrated that uncomplicated pregnancy represents a period of appreciable maternal gain over and beyond the fetal requirements (Hunscher, Hummel, Erickson and Macy, '35). Such a maternal reserve or 'rest material' is, in all probability, nature's means of preparing the mother

to carry on the physiological functions of the reproductive cycle without undue burden to her own body. It will be recalled that continuous observation during the final 145 days of gestation showed that L.R., on a generously chosen diet stored a total of 52.90 gm. of calcium, approximately 24 gm. of which were laid down in the fetus thus leaving a known reserve of 29 gm. for utilization or dissipation in the completion of the fourth reproductive cycle. Although the continuous balances were extended only from the tenth to fifty-third days postpartum during a period of heavy milk flow in which there was an observed maternal calcium loss of 21 gm. there still remained a known reserve of 7.90 gm. of calcium. As has been previously emphasized this woman laid down an abundance of the various inorganic elements during the last half of gestation over and beyond what was needed for the fetus and she had none of the common disturbances characteristic of this period. She experienced an excellent preparation through a building up of a generous maternal reserve in pregnancy which enabled her to complete the physiological functions of reproduction even to the secretion of a large quantity of breast milk with a physiological drain for which she was prepared. During the 43 days of lactation there was similarly a gain or loss of —1.57, + 22.27, + 34.83, + 16.55, — 15.80 and — 8.70 gm. of magnesium, sodium, potassium, chlorine, phosphorus and sulfur, respectively. If a mother is organically fit and she is given adequate medical care and advice during the entire reproductive cycle she should be able to carry out the ordinary functions of motherhood without a physiological burden to her own tissues, and at the same time endow her offspring with its rightful heritage—physical and nervous stability.

SUMMARY

Using the continuous metabolic balance method of study on one woman in her fourth reproductive cycle when consuming a generous food intake the mean daily balances of calcium, magnesium, sodium, potassium, phosphorus, sulfur and

chlorine were found to be 0.37 ± 0.19, 0.11 ± 0.05, 0.56 ± 0.53, 1.40 ± 0.72, 0.26 ± 0.16, 0.34 ± 0.13 and 0.60 ± 0.96 gm., respectively, during the final 145 days of pregnancy and — 0.48 ± 0.56, — 0.03 ± 0.15, + 0.47 ± 0.46, + 0.87 ± 1.06, — 0.37 ± 0.24, — 0.22 ± 0.18, + 0.39 ± 0.88 gm., respectively, from the tenth to fifty-third days of milk flow.

The twenty-eight acid-base balances during pregnancy showed a mean daily retention with standard deviation of 347 ± 246 cc. 0.1 N base. In lactation, because of losses of acid-forming elements, the base balance increased to 471 ± 423 cc. 0.1 N base per day.

The quantitative determination of the total accumulation of each individual element during the last half of pregnancy permitted a study of the comparative rates of gain of the elements and their relationships to one another with the advance of the reproductive cycle.

By the continuous observations there was no apparent change in the progressive gestatory needs as term approached.

On a maternal diet that was not only abundant in all the known nutritive essentials including minerals and vitamin D but in the proportions to be conducive to a high maternal storage the present woman was able to bear and breast feed an infant who showed no clinical or roentgenographic manifestations of rickets (Barnes, Cope, Hunscher and Macy, '34) although it never received any direct administration of vitamin D up to the eighth month of life other than that contained in its own mother's milk.

Providing a generous maternal reserve has been laid down in pregnancy over and beyond that necessary for fetal growth, the losses of the various elements that do occur in early lactation may have no apparent serious consequences as illustrated by this woman who has undergone frequent reproductive cycles during a period of 8 years.

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THE SIGNIFICANCE AND ACCURACY OF BIOLOGICAL VALUES OF PROTEINS COMPUTED FROM NITROGEN METABOLISM DATA

H. H. MITCHELL, WISE BURROUGHS AND JESSIE R. BEADLES
Division of Animal Nutrition, University of Illinois, Urbana

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Since the introduction by Thomas ('09) of a method of measuring the biological value of protein from nitrogen metabolism data obtained with human subjects, and the modification of this method and its adaptation to rats by Mitchell ('24 a), such methods have been extensively used in the study of the nutritive values of the proteins of food materials. A review of this work up to 1929 has been prepared by Mitchell and Hamilton ('29), and a more recent review was published by Boas-Fixsen ('35). Each investigator has generally introduced more or less extensive modifications of his own, either in technic, in method of calculation, or in application to animals of different age or species. Although in general the results obtained in these various laboratories have been mutually consistent, occasionally a wide variability has been observed among biological values obtained on the same food material (Chick, Boas-Fixsen, Hutchinson and Jackson, '35; Mason and Palmer, '35).

Many observers have commented on the shifting level of endogenous nitrogen excretion observed in rats upon which two or more determinations have been made, while some uncertainty as to the period of feeding required to reduce the protein catabolism to the endogenous level undoubtedly has arisen, particularly since the publication of the work of Ashworth and Brody ('33) on this question. The reality of a

level of endogenous nitrogen excretion possessing a definite biological significance, in particular a definite relationship to the demands of the body for nitrogenous compounds for the maintenance of the integrity of its tissues, is evidently a matter of doubt in the minds of many.

The factors determining the excretion of metabolic nitrogen in the feces are not generally agreed upon, in particular whether this excretion varies mainly in response to variations in the intake of dry matter, or to variations in body weight of the animal, or whether it is an individual characteristic of an animal, unaffected by body weight or food intake.

In defense of the theory underlying the nitrogen metabolism method of determining biological values of proteins, the work of Terroine and his associates (cited fully by Smuts) and of Smuts ('35) possesses great significance. This work reveals a remarkably close correlation between the endogenous excretion of nitrogen as ordinarily determined and the basal heat production, not only within a single species, but also among species varying in weight from the mouse to the pig. According to Smuts, an average of 2 mg. of endogenous nitrogen is excreted per calorie of basal heat. The reality of a constant and significant basal output of nitrogen would thus seem to be as well established as that of a constant and significant basal output of heat. And while conditions, both internal and external, are known to cause a shift in the value of the former, the situation is quite the same with reference to the latter; in particular close and prolonged confinement of an animal will lower its basal metabolism (Lusk and DuBois, '24; Mitchell and Haines, '27). Furthermore, Benedict ('28) has developed the thesis that the basal metabolism is a measure of vital activity and is subject to all those influences that depress or stimulate vital activity.

The factors to which the excretion of metabolic nitrogen in the feces is subject have been studied in some detail by Schneider ('35) and Mitchell ('34). It seems clear that, for levels of food intake compatible with a good determination of biological value, the predominant factor is the quantity of

dry matter consumed. The failure of other investigators to observe this relationship is not surprising whenever the experimental plan involves short collection periods with no use of feces markers.

The variability of individual determinations of the biological value of a protein is the resultant not only of technical error in method, but also of individual differences in protein utilization in metabolism. In thirty of the most recent determinations at this laboratory, involving groups of nine or ten rats each, the average variability, as measured by the standard deviations of the thirty groups of values, is 3.7. The individual standard deviations varied from 1.7 to 7.2, and tended to be larger for the smaller biological values. One may predict, therefore, that the average of ten biological values would possess a standard deviation of 1.2 ($3.7 \div \sqrt{10}$), and that successive averages of ten would in all probability fall within an interval of $\pm$ 2.4 from the grand average of all. The best test of this prediction is afforded by the experiments of Mitchell and Carman ('26), in which five determinations of the biological value of the proteins of patent white flour were made with five groups of ten rats each. The average group determinations were 52, 53, 51, 49 and 55, averaging 52. All averages fell within the interval 52 ± 3. This is a relatively low biological value. The proteins of dried skim milk have a much higher value, which has been assessed at 85 (Mitchell, '24 b), 84 (Mitchell and Carman, '26), 84 (Mitchell, Beadles and Keith, '26) and 82 (Fairbanks and Mitchell) in various publications from this laboratory. These instances illustrate, for a protein of low biological value as well as for one of high value, the reproducibility of results obtained by the nitrogen balance method as it is used in the Division of Animal Nutrition of the University of Illinois.

In this paper the authors will report the results of tests of this method to determine whether the biological values obtained by it possess an absolute significance, or at most merely a comparative significance, as they would if the reasoning upon which their calculation is based is unsound, and if

the significance attached to the endogenous urinary nitrogen and the metabolic fecal nitrogen is false. An illustration will also be given of the significance of small differences between average biological values.

The tests involve a comparison of biological values by the nitrogen balance method and of results of paired-feeding tests with the same rations. It seems unnecessary to give the details of the experimental technic in each case, since they may be found in many previous publications from this laboratory, for example, the paper of Beadles, Quisenberry, Nakamura and Mitchell ('33). The only deviations from past practices were, first, the use of rations containing 22 instead of 10 per cent of fat, in the hope that the change in consistency would be such as to minimize scattering by the experimental rats. Since this hope was not realized, all food portions after being weighed into the food cups were mixed with water to a thin consistency, an expedient that had the desired effect. In previous work, a level of 8 per cent of dietary protein has been adopted, but with the higher fat content of the diets, the protein content was raised from 8 to 9.2 in order to maintain constant the percentage (7.6) of protein calories in the diets. The second deviation from the usual procedure was the use of feces markers, either Fe_2O_3 or Cr_2O_3, to insure sharp separations of fecal collections.

RESULTS OF THE TESTS OF METHOD

I. Beef and peanut proteins

A comparison of the nutritive value of the proteins of raw peanuts (white meat only), and of the same sample of peanuts after roasting (heating to 400° to 450°F. for 30 to 35 minutes), by both the paired-feeding method and the nitrogen balance method, revealed a statistically significant, but numerically small, impairment due to heating. In the former experiment, ten pairs of rats, ranging in weight from 32 to 58 gm., were used. The feeding period lasted 5 weeks, during which time the rate of growth ranged from only 3 to 7 gm. weekly, due to the unpalatability of the raw peanut ration. After the termination of the experimental period, it was shown, by

doubling the daily supplements of yeast vitamin powder for five of the ten pairs, that the low food intakes were not the result of a deficiency of vitamin B or G.

The differences between pair mates with reference to total body weight gains during the 5-week test ranged from 0 to 4; in one pair the rat on the roasted peanut ration gained 1 gm. more than its pair mate, in two pairs the gains were equal, while in seven pairs the rat on the raw peanut ration gained more than its pair mate. These differences in outcome, while numerically small, possess a high statistical significance. Applying Student's method ('08) for the statistical analysis of

TABLE 1

True digestibility and biological value of the proteins of raw and roasted peanuts

RAT NO. AND SEX	RAW PEANUT PROTEIN		ROASTED PEANUT PROTEIN	
	True digestibility	Biological value	True digestibility	Biological value
1 ♂	97.7	61	95.8	55
2 ♂	99.2	60	98.0	54
3 ♂	96.1	56	94.4	47
4 ♂	95.9	63	96.8	59
5 ♀	97.2	56	97.1	51
6 ♀	99.5	63	96.2	61
7 ♀	98.6	59	96.5	57
8 ♀	97.2	56	95.0	54
9 ♀	96.6	44	95.0	62
10 ♀	95.9	61	96.7	58

paired experimental observations, the mean difference in gain is 1.80 gm., favoring the raw peanut protein, the standard deviation of differences is 1.72 gm., and the probability that fortuitous factors alone would produce as consistent a series of differences as those obtained is only 0.006. This probability is so small it may be neglected. The conclusion appears to be justified, therefore, that roasting has impaired the nutritive value of the peanut to a slight extent.

This impairment may relate to digestion or metabolism or both. A further analysis of the problem was made possible by the results of the nitrogen balance studies. The coefficients of true digestibility and the biological values obtained for the ten rats used in these studies are summarized in table 1. The

average coefficients of true digestibility (allowance being made for all metabolic products in the feces) for raw and roasted peanuts are 97.4 and 96.1, respectively, and the average biological values of the absorbed protein are 57.8 and 55.7, respectively. While these averages do not differ greatly, the differences are, with two exceptions in the first case and one in the second, consistent among the ten rats. Analyzed by Student's method, the probability that chance only determined the outcome is 0.0094 (M = 1.24, s = 1.29) with reference to digestibility, and 0.00005 (M = 2.1, s = 0.99) with reference to biological value. It may be concluded, therefore, that the impairment of the growth-promoting value of peanut protein during roasting, indicated in the paired-feeding test,

TABLE 2

True digestibility and biological value of beef round protein

RAT NO. AND SEX	TRUE DIGESTIBILITY	BIOLOGICAL VALUE	RAT NO. AND SEX	TRUE DIGESTIBILITY	BIOLOGICAL VALUE
1 ♂	100.0	79	6 ♀	100.0	81
2 ♂	98.0	77	7 ♀	100.0	79
3 ♂	100.0	75	8 ♀	100.0	79
4 ♂	99.4	81	9 ♀	99.5	77
5 ♀	100.0	76	10 ♀	99.3	73

relates both to digestion and to metabolism, although in both instances the impairment is numerically slight. The average biological value for raw peanut protein is in close agreement with the value of 59 obtained by Pian ('30) with six rats.

Immediately after the balance periods on raw and roasted peanuts, the ten rats were put upon a diet containing dried extracted beef round as the predominant source of protein. The results of this period are assembled in table 2. The average coefficient of true digestibility of the beef protein was 99.6 per cent, with six rats giving evidence of complete digestibility. The average biological value of the absorbed protein was 77.7, a value 34 per cent higher than that for raw peanut protein. In a previous publication from this laboratory, a biological value of 69 (Mitchell and Carman, '26) has been

reported for beef protein. However, such a variation is to be expected for meat proteins, because of a variable content of connective tissue proteins (Mitchell, Beadles and Kruger, '27).

A growth comparison of the value of beef protein and the protein of roasted peanuts was undertaken by the paired feeding method, using eight pairs of rats. These rats ranged in weight initially from 30 to 47 gm. and were paired with the usual care. At the end of the first week of feeding the superiority of the beef protein was clearly evident, and at the end of the third week, the advantage in total gain in weight averaged 8.75 gm. and was evident in each pair.

In order to obtain a quantitative measure of this superiority, which is impossible as long as the body weights of pair mates are unequal, the experimental procedure was changed starting with the fourth experimental week. The food intakes of pair mates were still equalized, but the beef protein ration was fed with varying proportions of a diet containing the same ingredients except that the dried extracted beef was replaced by starch. The proportion of this very low-nitrogen diet fed in varying amounts to the rats on the beef diet was increased until the rats on the peanut diet attained approximate weight equality with their pair mates, a result that was attained by the end of the fifth week, and then was continually readjusted to maintain this equality through the twelfth week of feeding, at about which time the experiment was terminated. The significant results of this modified paired-feeding test will be found in table 3.

The total gains in weight of the pairs varied widely for the 12 weeks of feeding, from 40 and 46 gm. for pair 7 to 119 and 112 gm. for pair 2, in close conformance with the variation in the total intake of food, but for the rats upon the same diet the gains were quite similar, averaging 80.5 gm. for the rats on the peanut diet and 81.9 gm. for the rats on the beef diet. The intake of food was identical for all pair mates, but the intake of nitrogen was consistently higher for the rats on the peanut protein, averaging 9.44 gm., as compared with 6.61

gm. for the rats on the beef protein. The ratio of nitrogen intakes of pair mates varied from 1.29 gm. of peanut nitrogen to 1 gm. of beef nitrogen, to 1.74 to 1. The average ratio was 1.46 to 1. This ratio means that 1 gm. of beef nitrogen in this experiment was as effective in promoting the functions of

TABLE 3

A comparison of the growth-promoting value of the proteins of roasted peanuts and of beef round by a modified paired-feeding method. All weights expressed in grams

	PAIR 1 ♂		PAIR 2 ♂		PAIR 3 ♂		PAIR 4 ♀	
	Peanut ration	Beef ration + N-free ration	Peanut ration	Beef ration + N-free ration	Peanut ration	Beef ration + N-free ration	Peanut ration	Beef ration + N-free ration
Total gain in weight	64	71	119	112	91	91	96	96
Total food consumed	621	621	822	822	705	705	745	745
Total nitrogen consumed	8.77	5.41	11.20	8.71	9.89	7.53	10.51	7.48
Ratio of nitrogen intakes	1.62	1	1.29	1	1.31	1	1.41	1
Activity, revolutions (× 1000)	376	561	340	274	481	869	413	240
Body length in millimeters	184	181	204	203	192	190	190	187

	PAIR 5 ♀		PAIR 6 ♀		PAIR 7 ♂		PAIR 8 ♂	
	Peanut ration	Beef ration + N-free ration	Peanut ration	Beef ration + N-free ration	Peanut ration	Beef ration + N-free ration	Peanut ration	Beef ration + N-free ration
Total gain in weight	97	97	5	56	40	46	82	86
Total food consumed	723	723	538	538	563	563	664	664
Total nitrogen consumed	10.20	7.63	7.60	5.53	7.94	4.56	9.38	6.01
Ratio of nitrogen intakes	1.34	1	1.37	1	1.74	1	1.56	1
Activity, revolutions (× 1000)	397	317	543	1067	1074	560	1029	468
Body length in millimeters	190	188	166	165	172	167	189	184

maintenance and of growth as were 1.46 gm. of peanut nitrogen.

A part of this difference is the result of the poorer digestibility of peanut nitrogen as compared with beef nitrogen, the coefficients of true digestibility being 96.1 and 99.6, respectively (see above).[1] But, on the other hand, the metabolic

[1] The digestibility of the energy of the peanut and beef rations was very nearly the same, averaging (for four rats in each case) 96.9 and 97.8, respectively.

nitrogen of the feces excreted per gram of dietary nitrogen was greater for the rats on the beef ration, because of the lower percentage of nitrogen in the latter diet. When these two factors are taken into account, the latter factor by evaluating the metabolic fecal nitrogen at 1.34 mg. per gram of dry matter consumed (on the basis of the nitrogen balance studies), the ratio expressing the nutritive equivalence of peanut nitrogen and beef nitrogen is practically unchanged, being 1.47 to 1.

In spite of the equality of gains between pair mates, there was a slight, though statistically significant, difference with respect to body length, measured from nose to root of tail, as the figures in table 3 show. In each pair the final body length of the rat on the peanut ration was greater than the body length of its pair mate on the beef protein ration. The average difference amounted to 2.75 mm. while the standard deviation of differences was only 1.48 mm. The probability that chance alone was responsible for this outcome is so small (0.0009) that it must be considered a dietary effect.

When the experiment was planned it was realized that if a large difference in the nutritive values of peanut protein and of beef protein existed, a differential effect of the two diets upon the muscular activity of the animal may occur. In unpublished experiments, one of us (H.H.M.) has shown that the mere restriction of the food intakes of a rat will almost invariably increase its voluntary activity as measured in a revolving cage. If the rats on the peanut protein ration were more active than their pair mates, they would gain less per unit of food energy consumed. Consequently, the feeding experiment was conducted in a battery of sixteen revolving cages, with a revolution counter for each cage. The total revolutions for each rat for the entire experiment are given in table 3, expressed in thousands. There was no consistent relation between the activity of pair mates on the two experimental rations, the average number of revolutions being 582,000 for the rats on the peanut ration and 545,000 for the rats on the beef ration. It may be concluded, therefore, that the results

of the growth experiment were not vitiated in any way by a differential effect of the diets upon voluntary activity.

Although it is commonly believed that equal gains on equal intakes of food denote equal nutritive effects, this is not necessarily true, since the tissue added during growth may vary in composition, particularly with reference to the content of moisture, fat and protein. The reality of this supposition has been proven in other experiments (Beadles, Quisenberry, Nakamura, Mitchell, '33; McClure, Voris and Forbes, '34).

TABLE 4

The contents of nitrogen and energy in the carcasses of four pairs of rats used in comparing the growth-promoting value of peanut protein and beef protein

PAIR NO.	RATION	EMPTY BODY WEIGHT, GM.	NITROGEN CONTENT		ENERGY CONTENT	
			In per cent	In grams	Per gram calories	Total calories
1	Peanut	120.8	2.99	3.61	1.91	231
	Beef	124.8	2.47	3.08	2.82	352
2	Peanut	176.8	2.86	5.06	2.36	417
	Beef	171.1	2.91	4.98	2.52	431
3	Peanut	142.5	3.04	4.33	2.27	323
	Beef	142.6	3.12	4.45	1.97	281
4	Peanut	144.8	3.11	4.50	2.26	327
	Beef	144.6	2.92	4.22	2.29	331

In the present test it seemed advisable to investigate this possibility, and accordingly the first four pairs of rats were subjected to analysis to determine their contents of nitrogen and of gross energy (heat of combustion). The results are summarized in table 4.

There are no consistent differences between pair mates, either with respect to nitrogen or energy content, and, with the unexplained exception of pair 1, there are no great differences between pair mates. These analyses, therefore, afford no grounds for doubting that the two dietary regimes compared in this test actually did produce identical nutritive effects as well as identical gains in body weight.

It may be concluded, therefore, that this modified paired feeding experiment has provided valid evidence that, in the experimental rations fed, 1 gm. of absorbed beef nitrogen has proven to be the nutritive equivalent of 1.47 gm. of absorbed peanut nitrogen in replacing endogenous losses of nitrogen (maintenance) and in promoting growth. The nitrogen balance method yielded biological values for these two mixtures of protein of 77.7 and 55.7, respectively. These values indicate that 1.29 gm. ($1 \div 0.777 = 1.29$) of beef nitrogen cover the body's requirement for 1 gm. of nitrogen, while for the same purpose 1.80 gm. ($1 \div 0.557 = 1.80$) of peanut nitrogen are required. Hence, by these tests 1 gm. of beef nitrogen is the equivalent of 1.40 gm. ($1.80 \div 1.29 = 1.40$) of peanut nitrogen, an equivalence ratio approximating closely to that yielded by the paired feeding technic, i.e., 1 to 1.47 gm. The somewhat greater relative value of beef nitrogen indicated by the latter method may be the result of an enhanced biological value at the lower level of feeding employed, i.e., 0.97 per cent, as compared with 1.40 per cent for the peanut nitrogen. The remarkable agreement between the two technics for assessing the nutritive value of proteins supports the validity of both, and particularly supplies striking confirmation of the somewhat controversial theory upon which the calculation of biological values rests.

II. Beef and pecan proteins

A somewhat similar comparison of the results of the paired-feeding and the nitrogen balance methods was made using beef proteins and pecan proteins. Preliminary digestion experiments indicated a marked difference in the true digestibility of these protein mixtures, beef protein being digested practically completely, while pecan protein was digested only to the extent of about 70 per cent. Hence, the experimental rations were made to contain the same percentage of digestible protein, 9.4 per cent. The gross energy contents of all rations were equalized at 4.85 calories per gram. However, the digestible energy content of the beef ration was 4.66

calories per gram, while that of the pecan ration was 4.32 calories per gram.

In the nitrogen balance experiment the standardizing ration, containing 4 per cent of whole egg, was fed in the second period, the test rations in the first and third periods. Five of the ten rats received the beef ration in period 1, and five the pecan ration. In period 3 the test rations were reversed for the two groups of rats. The rats were all males and weighed initially from 53 to 65 gm. Feces markers, ferric or chromic oxides, were used to separate the period collections.

TABLE 5

True digestibility and biological value of the proteins of round beef and pecan nut

RAT NO.	BEEF ROUND PROTEINS		PECAN NUT PROTEINS	
	True digestibility	Biological value	True digestibility	Biological value
17	100.0	66	73.2	61
19	100.0	71	70.3	63
21	100.0	71	66.3	58
23	100.0	75	70.5	61
25	100.0	75	71.6	64
18	100.0	80	72.9	58
20	100.0	80	71.7	60
22	100.0	78	70.3	59
24	100.0	73	67.7	57
26	100.0	77	72.6	57
Average	100.0	75	70.7	60

The results of the test are summarized in table 5. No evidence was obtained of the excretion of beef nitrogen in the feces of any of the ten rats, so that a coefficient of true digestibility of 100 was assigned in each case. The pecan protein again showed a low true digestibility, averaging 70.7 per cent. The biological values averaged 75 for beef protein and 60 for pecan protein. These values indicate that 1.33 gm. ($1 \div 0.75 = 1.33$) of digestible nitrogen from beef cover the body's requirement for 1 gm. of nitrogen, while for the same purpose 1.67 gm. ($1 \div 0.60 = 1.67$) of nitrogen from the pecan nut are required. Hence, 1 gm. of absorbed beef nitrogen is the nutritive equivalent of 1.26 gm. ($1.67 \div 1.33 = 1.26$) of absorbed pecan nitrogen.

In the growth experiment, eight pairs of rats weighing initially from 50 to 68 gm. were used. During the first 4 weeks of the test, the two rations were fed in equal amount to the two rats in each pair. At the end of this time a distinct difference in gain between pair mates in favor of the beef ration was noted, the mean difference being 7.6 gm., the standard deviation of differences 2.40 gm., and the probability of a fortuitous outcome less than 0.0001. Since the content of protein rather than of digestible energy was the factor limiting growth in both rations, and since the digestible nitrogen content of both rations was the same, this difference in gain may be attributed to the superior biological value of beef proteins.

From the end of the fourth week of the test to the end of the experiment the beef ration was diluted with a nitrogen-free ration until the body weights of pair mates were equalized, the total food intake of pair mates being kept the same. This equalization of body weights of pair mates was accomplished in 2 weeks for six pairs of rats, and in 5.5 weeks for two pairs. At the end of the experiment all rats were killed with ether, the contents of the alimentary canal were removed and the empty carcasses weighed, frozen solid, ground finely in a hand mill and analyzed for nitrogen. The gross energy content of four pairs of rats was determined with the bomb calorimeter. The results of these analyses are given in table 6.

Although the weights of pair mates were equalized in the last weeks of the experiment, the empty weights of the rats on the pecan ration were found to be smaller in all pairs than the weights of the corresponding rats on the beef ration. The percentages of nitrogen in the carcasses of pair mates, as well as the contents of energy in the four pairs examined, averaged almost the same. However, the absolute weights of nitrogen in the carcasses of beef protein rats exceeded those of the carcasses of the pecan protein rats in seven of the eight pairs, the average difference amounting to 77 mg. of nitrogen.

The final calculations from the paired-feeding experiment are given in table 7. The net intake of nitrogen of each rat is estimated by subtracting from the intake of digestible nitrogen the metabolic nitrogen of the feces, assessed at 1.49

TABLE 6

The contents of nitrogen and energy in the carcasses of rats used in comparing the growth-promoting value of beef protein and pecan protein

PAIR NO.	RATION	EMPTY BODY WEIGHT, GM.	NITROGEN CONTENT		ENERGY CONTENT	
			In per cent	In grams	Calories per gram	Total calories
1	Beef	99.2	2.94	2.92	....	....
	Pecan	95.8	2.78	2.66	....	....
2	Beef	110.6	2.99	3.31	2.62	290
	Pecan	109.7	3.01	3.30	2.65	291
3	Beef	105.0	3.01	3.16	2.36	248
	Pecan	103.9	3.18	3.30	2.26	235
4	Beef	95.1	2.94	2.80	....	....
	Pecan	92.4	2.94	2.72	....	....
5	Beef	97.0	3.25	3.15	....	....
	Pecan	95.6	3.08	2.94	....	....
6	Beef	101.2	3.06	3.10	....	....
	Pecan	97.2	3.13	3.04	....	....
7	Beef	97.5	3.09	3.01	2.05	200
	Pecan	96.8	3.02	2.92	2.04	197
8	Beef	96.0	3.15	3.02	2.22	213
	Pecan	93.4	3.19	2.98	2.33	217
Averages						
	Beef		3.054	3.059	2.31	238
	Pecan		3.041	2.982	2.32	235

mg. per gram of food, the average result of the standardizing period in the nitrogen balance experiment. A correction is then applied because of the differences in nitrogen content of pair mates at the end of the experiment (see table 6). Assuming that the rats contained 3 per cent of nitrogen at the begin-

ning of the experiment, it appears from average figures that each gram of nitrogen stored required 2.41 gm. of net nitrogen from the beef ration and 3.01 gm. of nitrogen from the pecan

TABLE 7

The nutritive equivalence of the nitrogen of beef round and of the pecan nut, according to the paired-feeding tests

PAIR NO.	RAT NO.	RATION	FOOD INTAKE	DIGESTIBLE NITROGEN	METABOLIC NITROGEN IN FECES	NET INTAKE OF NITROGEN	DIETARY NITROGEN TO CORRECT FOR DIFFERENCE IN NITROGEN RETENTION	CORRECTED NET INTAKE OF NITROGEN	RATIO
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
1	1	Beef	244 (17)[1]	3.76	0.39	3.37	0	3.37	1
	2	Pecan	261	4.08	0.39	3.69	+ 0.78	4.47	1.33
2	3	Beef	331 (101)	5.10	0.69	4.41	0	4.41	1
	4	Pecan	432	6.66	0.69	5.97	+ 0.03	6.01	1.36
3	5	Beef	323 (106)	4.97	0.68	4.29	+ 0.34	4.63	1
	6	Pecan	429	6.63	0.68	5.95	0	5.95	1.29
4	7	Beef	229 (12)	3.53	0.36	3.17	0	3.16	1
	8	Pecan	241	3.77	0.36	3.41	+ 0.24	3.64	1.15
5	9	Beef	221 (21)	3.40	0.35	3.05	0	3.05	1
	10	Pecan	242	3.79	0.35	3.44	+ 0.63	4.07	1.33
6	11	Beef	241 (28)	3.71	0.38	3.33	0	3.33	1
	12	Pecan	269	4.21	0.38	3.83	+ 0.18	4.01	1.20
7	13	Beef	238 (10)	3.66	0.38	3.28	0	3.29	1
	14	Pecan	248	3.88	0.38	3.50	+ 0.27	3.78	1.15
8	15	Beef	243 (17)	3.74	0.39	3.35	0	3.36	1
	16	Pecan	260	4.08	0.39	3.69	+ 0.12	3.81	1.13
Averages		Beef rats				3.53			1
		Pecan rats				4.19			1.24

[1] Amount of nitrogen-free ration consumed.

ration. The correction represents an addition to the net intake of nitrogen of the pair mates containing the smaller final content of nitrogen equal to its deficit of nitrogen multiplied

by 3.01, in the case of a rat on the pecan ration, or by 2.41, in the case of a rat on the beef ration. The corrected net intakes of nitrogen, given in the next to the last column of table 7, would presumably induce equal storages of nitrogen in growing rats. The ratios of these intakes, taking the beef ration rat as a base, are given in the last column. These ratios average 1 to 1.24, and represent the nutritive equivalence of beef and pecan nitrogen.

This average ratio is very close to that obtained in the nitrogen balance studies, i.e., 1 to 1.26. Since the calculations from the growth experiment do not involve any assumptions concerning the magnitude, the constancy, or the significance of the endogenous losses of nitrogen from the body, while those from the nitrogen balance experiments are based in large part upon what appear to be reasonable assumptions in regard to these losses, the approximate identity of the results of both methods constitutes a striking confirmation of the accuracy of these assumptions. Hence, it may be concluded that the biological values determined by the nitrogen balance method possess an absolute as well as a relative significance.

CONCLUSIONS

Biological values of a given protein, as determined by the nitrogen balance method as it is used in this laboratory, exhibit an average standard deviation of 3.7. This statistic tends to be larger the smaller the biological value.

The statistical significance of a difference in biological value between two protein mixtures of less than 4 per cent may be clearly demonstrated by the nitrogen balance method.

The relative nutritive equivalence of two protein mixtures for maintenance and growth is practically the same when evaluated by the nitrogen balance method or by the paired-feeding method supplemented by carcass analyses. Hence, the assumptions underlying the calculation of biological values by the method developed in this laboratory are substantially correct, and the biological values possess an absolute as well as a relative significance.

The biological value of beef round protein, providing 7.6 per cent of the calories of the test ration, averages 76, that of raw and roasted peanuts 58 and 56, respectively, and that of the pecan nut, 60. Roasting of the peanut according to the usual commercial practice depresses both the digestibility and the biological value of its protein, though only to an inappreciable extent.

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RICKETS IN RATS

XV. THE EFFECT OF LOW CALCIUM-HIGH PHOSPHORUS DIETS AT VARIOUS LEVELS AND RATIOS UPON THE PRODUCTION OF RICKETS AND TETANY [1]

ALFRED T. SHOHL

WITH A NOTE BY S. B. WOLBACH

Department of Pediatrics, Harvard Medical School, and The Infants' and The Children's Hospitals, Boston, Massachusetts

ONE FIGURE

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The process of deposition of minerals in osteoid tissue is usually called calcification, and it was quite natural, therefore, that early investigations focused upon calcium metabolism and calcium deprivation. The study of E. Voit, in which puppies were fed horse meat may be mentioned as an example. Such work antedated both the classic description of the histologic pathology of rickets and the knowledge of the vitamin factors. A review of this literature is given by Goldblatt ('31). Since the investigations of Mellanby, and McCollum, Park and associates, and Sherman and Pappenheimer, the principal interest has centered upon rickets produced by high calcium low phosphorus diets. These produce an advanced degree of rickets in rats and the histologic pathology and serum values of calcium and inorganic phosphorus closely approach those found in ricketic infants. These diets have been employed almost universally in the studies for vitamin tests.

[1] Read before the American Society of Biological Chemists at Detroit, April, 1935.

Since the advent of vitamin D comparatively little attention has been given to low calcium diets. The Baltimore investigators (McCollum et al., '21, '22; Shipley et al., '22) reported that rickets could be produced in rats by diets low in calcium and moderate in phosphorus, findings which have been confirmed by Park et al. ('23) and Jones and Robson ('33). In fact, they defined this as a second type of rickets similar to that found clinically associated with low serum calcium and tetany. In all these studies it was necessary to have in addition to a deficiency of vitamin D or ultra-violet light, a disproportion between the calcium and phosphorus in the diet. In this respect, then, rickets in rats differed essentially from that found in dogs and infants, in which the dietary Ca/P approximated that found in milk (Ca/P = 1.3).

The normal Ca/P has been defined as from 2/1 to 1/2 and it has been shown that such diets will not cause rickets in rats but will cure rickets after it has been produced. In a previous communication (Brown, Shohl et al., '32) we demonstrated that the ratio of Ca/P alone was inadequate to define the ricketogenic properties of a diet and that the absolute amounts or concentrations must also be given. When the ratio was fixed, rickets was more severe at low levels and less severe or absent at higher levels.

Serum calcium and phosphorus studies by Park, Guy and Powers ('23), Bethke and associates ('32), Kramer and Howland ('32), Brown and associates ('32), have shown that the blood serum values for calcium and phosphorus parallel those of the diet. In the last three of these studies the relation to the dietary levels as well as the ratios is clear. When the Ca/P of the diet is constant, as the absolute amounts ingested increase both calcium and phosphorus of the serum are raised.

The ash content of fat-free bone has been used to give insight into the amount of mineral deposition and thus to give a quantitative value of the degree of rickets. Such an evaluation can be made only roughly by other methods. Bethke et al. ('32) have correlated increase in bone ash with the amounts of calcium and phosphorus intake at a given ratio.

He found slight increases in bone ash with low calcium high phosphorus diets parallel to the increase in salt concentration. But no one has correlated serum values and bone ash with both ratios and levels of intake and histologic pathology in this type of rickets.

Binger ('17) has shown that intravenous phosphate caused a lowered serum calcium and tetany in dogs when the solutions were alkaline or neutral, and low serum calcium but no tetany when they were acid. Salvesen, Hastings and McIntosh ('24) were able to show that high phosphate ingestion caused low serum calcium and tetany. Some infants develop tetany without ever passing through a phase of low phosphorus rickets but show low calcium and normal or increased phosphate in the serum (Gerstenberger et al., '30). In the healing of rickets, infants and rats may develop tetany which is associated with an increased serum phosphate (Shohl et al., '32). The effect of low calcium intakes at various levels and ratios of calcium and phosphorus in the diets of rats upon the production of rickets and concomitant tetany has not been described.

It seemed necessary, therefore, to extend and supplement our former investigations of the high calcium low phosphorus diets to include the low calcium high phosphorus diets and thus in a systematic fashion to complete a survey of the various Ca/P ratios and levels attainable with natural foodstuffs. It was not our purpose to include diets in which the calcium or phosphorus was so low as to occur in traces only. Such calcium or phosphorus starvation requires the use of specially purified ingredients and constitutes a separate problem. Neither was it our purpose to add so much salt that nutritive failure would ensue.

PLAN OF EXPERIMENT

Albino rats from our colony were weaned at 21 days and placed upon the experimental diets at 28 days for a period of 21 days. Four to six animals were fed each diet. The animals were weighed weekly.

Diet. The diet of the breeding and lactating animals was Sherman diet B which consists of two-thirds ground whole wheat and one-third whole milk powder, with $\frac{2}{3}$ per cent of NaCl. Lettuce and beef liver were given about twice a week. The experimental diets consisted of alterations of Steenbock and Black's diet no. 2965 with the $CaCO_3$ omitted (diet A). When lower phosphorus values were desired Hess and Sherman's modification was used (diet M). The former diet consists of 79 per cent corn, 20 per cent gluten and 1 per cent NaCl, and the latter diet is the same except that corn meal is substituted for ground corn.

The calcium contents of these basal rations were 0.06 and 0.05 per cent, respectively. The phosphorus contents were 0.29 and 0.12 per cent. To these were added appropriate amounts of $CaCO_3$ or KH_2PO_4 or both to obtain the desired ratios and levels shown in table 1. The values have been rounded off to even figures. The calcium was varied from 0.06 to 1.0 per cent and the phosphorus from 0.12 to 2.0. Above this level of phosphorus the salt effect is so great that the animals lose weight and die. These diets vary not only in calcium and phosphorus but also in acidity, carbonate and potassium, and hence in any derived values such as Na/K, K/Ca or CO_3/PO_4.

The acidities are also given in the table calculated according to the method of Sherman and Sinclair, except that the phosphorus is given a valence of 1.8. The acidities of the basal diets are +70 and +12 cc. 0.1 N acid per 100 gm. of diet. The additions of calcium and phosphate cause variations from —365 to +495 cc. The greatest acidities are found in the diets to which most phosphate was added.

The effects of the K/Na or K/Ca have not been shown to be of importance in rickets but have been widely studied in regard to tetany. The conditions selected were those most favorable to the production of tetany, except for the acidity: low calcium, high phosphate, high K/Ca and high K/Na (Seekles and Sjollema, '33). Preliminary experiments (Brown, '30) in which Na_2HPO_4 was used and which did

not involve increased acidity or the last two factors did not produce frank tetany.

Methods. The galvanic electrical reactions were determined weekly (Shohl and Bing, '28). At the end of 21 days on the experimental diet the animals were x-rayed. The use of a suitable rat board (Shohl, '31) permitted as many as twenty pictures in 15 minutes, of unanesthetized animals, without exposure to the operator and with the bones in constant position. The rats were bled to death under light ether anesthesia, and autopsied. The calcium and phosphorus of the pooled serum were analyzed by the method of Fiske and Subbarrow ('25) and Fiske and Logan ('31). When sufficient serum remained, total nitrogen was determined by the Kjeldahl method. The leg bones were dissected free of tissue and the femurs of one leg were weighed, dried, extracted with alcohol and ether, and incinerated to determine the ash content. The average of the individual determinations or the group value was used. The bones of the other leg were reserved for histological examination by Dr. S. B. Wolbach, who has kindly furnished a note on his findings.

The criteria of the effect of the various diets employed were: 1) electrical reactions, 2) blood serum analyses for calcium and phosphorus, 3) the per cent of ash of the fat-free femurs, 4) roentgenograms and 5) histological examination of the bones.

RESULTS

The experiment proceeded with no known errors. The gain in weight in most groups averaged about 4 to 7 gm. per week. In the high salt groups especially with high phosphorus and low calcium the gain was 1 to 2 gm. or absent. In a few animals slight losses occurred. The feces were normal; no diarrhoea occurred with the high phosphorus diets. The animals appeared normal and did not seem unduly nervous.

1. Electrical reactions. No animals were seen in general convulsions of tetany. Two individuals were observed in a slight tremor and one of these in carpopedal spasm. Both of these animals were in the groups fed with diets in which

the Ca/P was 1/4. The amounts of current which caused neuromuscular contraction in the animals which showed tremor were low enough to class the response as tetany. All of the other animals at this ratio, Ca/P = 1/4, or with diets containing still greater amounts of phosphorus showed responses to electric stimuli which must be classed as latent tetany. The effects of Ca/P = 1/2, or lower phosphorus gave

TABLE 1

Effect of varying levels and ratios of calcium and phosphorus of the diet

COMPOSITION OF DIET					BONE ASH [2]	BLOOD SERUM		DEGREE OF RICKETS [3]	
Diet	Ca/P	Calcium	Phosphorus	Acidity		Calcium	Phosphorus	X-ray	Histology
		per cent	*per cent*	*cc. 0.1 N* [1]	*per cent*	*mg. per cent*	*mg. per cent*		
R	2	0.25	0.12	— 78	38	8.8	5.2	++	++
S	2	0.50	0.25	—130	45	7.5	5.8	+	+
X	2	1.00	0.50	—365	46	10.0	7.6	—	Not done
N	1	0.12	0.12	— 18	40	4.8	8.8	++	++
K	1	0.25	0.25	— 30	46	6.0	11.4	+	+
L	1	0.50	0.50	— 65	48	7.6	10.0	—	—
Q	1	1.00	1.00	—195	50	9.7	11.0	—	—
M	1/2	0.06	0.12	+ 12	27	5.0	7.8	+++	+++
F	1/2	0.12	0.25	+ 40	41	5.6	9.0	+	+
G	1/2	0.25	0.50	+ 45	46	6.2	11.6	—	±
O	1/2	0.50	1.00	+ 85	46	9.3	9.3	—	—
U	1/2	1.00	2.00	+ 45	48	8.2	10.6	—	—
A	1/4	0.06	0.25	+ 70	28	4.6	9.4	++	++
B	1/4	0.12	0.50	+105	36	4.8	11.0	++	++
I	1/4	0.25	1.00	+195	47	5.6	11.4	—	—
H	1/4	0.50	2.00	+325	42	...	11.2	—	—
C	1/8	0.06	0.50	+135	35	4.2	10.7	+	++
D	1/8	0.12	1.00	+255	35	4.1	9.8	—	++
P	1/8	0.25	2.00	+305	40	6.3	15.2	—	—
E	1/16	0.06	1.00	+285	32	3.9	7.6	—	++
V	1/16	0.12	2.00	+495	36	3.6	11.6	—	—

[1] Calculated per 100 gm. of diet. + = acid; — = base.

[2] Ash of the fat-free femurs.

[3] Plus signs indicate rickets; minus signs no rickets.

responses which were within normal limits. By this method, no difference could be detected as to the absolute amounts of calcium and phosphorus at the same ratio. If such differences occurred they were within the error of the method.

2. Calcium and inorganic phosphorus of the blood serum. In general, the blood reflects the proportions of calcium to phosphorus in the diet. This has previously been well established by Steenbock, by Bethke and associates ('32), and Kramer and Howland ('32). However, when the data given in table 1 are examined, it is at once seen that not only the ratio but also the levels or concentrations have a definite effect. *As the salt level increases at any ratio both the calcium and inorganic phosphate increase.*[2] In Bethke's study ('32) these effects are minimal but from Kramer and Howland's data ('32), though not analyzed from this viewpoint, the same conclusions must be drawn. This is due to the fact that the former did not vary his concentrations as widely as did the latter.

3. Bone ash. The data given in table 1 also clearly show that both the ratio and level of calcium and phosphorus determine the mineralization of bone. As the phosphorus is increased in proportion to calcium the bone ash is diminished. Furthermore, the effect of the levels is marked. When the concentrations are low the bone ash is low *and as the levels increase at a given ratio the bone ash increases.*[2] Our findings are in agreement with those of Bethke for the diets he used, but unfortunately he did not use diets low enough in calcium and phosphorus to bring out the relations shown here.

4. X-ray. The findings of the x-ray pictures are given in table 1 and graphed in figure 1. For the sake of completeness we have included in the figure the data from our high calcium low phosphorus studies (Brown et al., '32) to cover the entire range of diets available. The shaded area includes all changes from the normal regardless of the degree of rickets. The three types of shadings are simply a convenient separation

[2] If one wishes to take the trouble to rearrange according to levels of Ca and/or P in the diet (not tabled), this effect is brought out more strikingly.

into the rickets produced by high calcium low phosphorus, low calcium low phosphorus and low calcium high phosphorus diets respectively. The unshaded area represents the field in which no rickets was obtained. The degree of rickets indicated by the number of plus signs is only roughly quantitative.

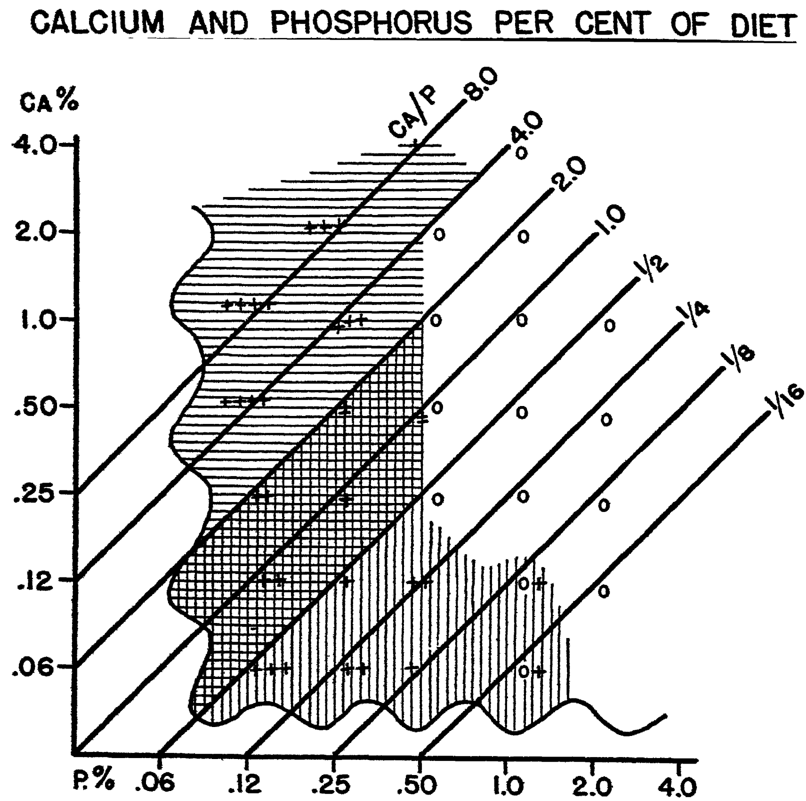

Fig. 1 For explanation see text.

Thus it can be seen that each of these three categories includes diets which are ricketogenic or non-ricketogenic depending upon the absolute amounts of calcium and phosphorus contained.

The graph was drawn independently of the histological findings and the conclusions differ from Doctor Wolbach's interpretation only in regard to the groups fed diets D and E.

These have a very narrow metaphysis, but histologically show rickets. The histological examination places these diets in the ricketogenic group, and the graph shows them in this category.

5. *Bone histology.*[3] The upper end of the tibia was used for histological study. The technic was fixation in 10 per cent formalin solution, decalcification in 5 per cent HNO_3 in 70 per cent alcohol, celloidin embedding and staining with hematoxylin and eosin. One or more representatives of each diet listed in table 1 were studied, with the exception of diet X, as follows: diets K, L and F, one rat each; diets R, S, Q, G, O, U, B, I, H, C, P, E, D and V, two rats each; diets N, M and A, four rats each.

The important histological criteria of rickets arise from disturbances in endochondral formation of bone and from failure of bone matrix or osteoid to calcify in all locations. For the purposes of this study we have restricted our observations to the epi-diaphysial regions, because most of the bones had been partially denuded of periosteum before fixation.

In brief review, the sequences in enchondral bone formation which are disturbed in rickets are as follows. The epiphyseal cartilage at the upper end of the tibia in normal white rats of the age employed consists of a narrow plate of cartilage firmly supported by bone on the epiphyseal side and uniformly penetrated by blood vessels of capillary dimensions on the diaphysial side. Very little evidence of growth is present on the epiphyseal side where bone is closely applied in the form of transverse trabeculae or a thin fenestrated plate. Growth is accomplished by continuous proliferation of cartilage cells, arranged in columns, on the epiphyseal side and simultaneous degeneration of the matured cells on the diaphysial side. The cavities occasioned by the degeneration and disappearance of the cartilage cells at the diaphysial end of the columns are invaded by capillaries accompanied by cells (osteoblasts) which are responsible for the deposition of bone matrix upon the exposed cartilage matrix.

[3] By Dr. S. B. Wolbach.

Growth of bone by enchondral bone formation is thus achieved by a continuously retreating gap in the continuity of tissues, maintained on the epiphyseal side by continuous renewal of cartilage cells and on the diaphysial side repaired by vascular outgrowth comparable to repair of any defect of tissues by the process of organization or granulation tissue formation. In normal growth there presents on the diaphysial side of the narrow epiphyseal cartilage a continuous layer of clear or empty cartilage cells forming an almost straight line.

The first histological evidence of rickets is the absence in whole or in part of the layer of clear cells and the consequent absence of ingrowth of capillaries. Slight degrees of rickets are manifested by a moderate increase in width of the epiphyseal cartilage presenting an irregular border on the diaphysial side. This irregularity is due to the fact that the cessation of degenerative sequence in the cartilage cells does not take place simultaneously over the diaphysial border. The width of the epiphyseal cartilage continues to increase because of the continued activity of the proliferative zone and the survival of the cells on the diaphysial side. The first histological evidence of repair following corrections of the diet is the presence of cleared or degenerated cells on the diaphysial border of the cartilage, an effect visible at the end of 24 hours and accompanied by extensive vascular penetration within 48 hours (unpublished experiments of S.B.W.).

In rickets, after the cessation of the degenerative sequences of the cartilage cells, calcification of the cartilage matrix ceases, and osteoid material (bone matrix) accumulates around the capillaries of the diaphysis adjacent to the cartilage. In advanced rickets the noncalcified cartilage of the diaphysial border is often transversely stratified in places, evidently a mechanical effort from weight bearing. Osteoid material increases in amount with the duration of the dietary deficiency and, being noncalcified, is molded by the pressure of weight bearing. In long continued rickets there is disappearance of the cancellous bone of the diaphysis and marked

resorption of cortical bone. The degree or severity of rickets may be recorded on the basis of the prominence of anatomical changes demonstrable by roentgenograms or histological study. The latter is the more accurate. Obviously two factors enter into the production of the pathological picture; the duration of the deficient diet and the degree of the deficiency. In the present study the time element was constant so that the extent to which normal sequences were held in abeyance, with consequent increase of width of cartilage and accumulation of osteoid, may be regarded as a roughly quantitative means of measurement of the effect of the rickets producing diet.

No histological evidence of rickets was found in rats fed on diets Q, O, U, I, H, P, V, and in one rat of two examined fed on diet G. The slightest histological effect of rickets manifested by irregular cessation of penetration of the cartilage by capillaries and slight irregularity in the thickness of the cartilage as a whole, was found in the one rat examined fed on diet L.

Slight rickets, characterized by markedly diminished penetration of the cartilage by capillaries, considerable to marked irregular thickening of the cartilage as a whole, persistence of cartilage cells in the primary bone trabeculae (primary spongiosa), no stratification of cartilage and no osteoid was found in rats fed on diets K and B, also in one of two rats examined fed on diet S, and in one of two rats examined fed on diet G.

Moderate rickets, characterized by complete or almost complete absence of penetration of cartilage cells by capillaries, marked thickening of the cartilage as a whole, marked incorporation of cartilage cell groups in the primary bone trabeculae (primary osteoid), traces of stratification of cartilage and small amounts of osteoid were found in rats fed on diets R, N, A and C, also in one of two rats examined fed on diet S, and in one of four rats examined fed on diet M.

Severe rickets, characterized by complete absence of penetration of cartilage cells by capillaries, marked thickening of

the cartilage as a whole, marked incorporation of groups of cartilage cells in the primary spongiosa, marked stratification of cartilage and large amounts of osteoid were found in a rat fed on diet F, and in three of four rats examined fed upon diet M.

The histology found in the rats fed upon diets D and E requires special mention, as the presence of rickets was not demonstrated by the roentgenograms. Two rats were examined from each of these diets. In both instances there was almost complete absence of penetration of cartilage cells by capillaries, marked incorporation of cartilage cells in the primary bone trabeculae (primary spongiosa) and considerable amounts of osteoid present in proximity to the cartilage. In both instances the epiphyseal cartilage was narrow as compared with all other rats with evidences of rickets. In the rats of diet D the width was slightly greater than normal. In the rats of diet E the width was not greater than normal and in one of these it was narrower than the average width found in control rats.

Discussion. The prominent morphological changes in experimental rickets must be regarded as the consequence of retardation of normal sequences in the growth of bone. Two apparently separate processes are involved, one the failure of the epiphyseal cartilage cells to complete their cycle of growth, maturation and degeneration, and the other the failure of osteoid matrix to calcify. The proliferative activities of cartilage cells, capillaries and osteoblasts are not inhibited. The histological picture, typical of rickets, indicates that the proliferative activities of these tissues are not retarded and in some instances suggests acceleration. In the rats fed on diet E the narrow epiphyseal cartilages did indicate a marked retardation of rate of growth of the cartilage cells, but more study will be required to determine whether an interpretation on a purely chemical basis should be attempted.

The histological study of this series clearly proves that the effects of the various diets employed are essentially similar. The differences are wholly quantitative and may be estimated

roughly on the basis of the extent to which normal sequences, essential to bone formation, have been held in abeyance. Histological examination may reveal definite evidences of rickets in the absence of grossly demonstrable changes in the epiphyseal cartilage.

DISCUSSION

The findings in this study, showing the interrelationship of both the ratio and absolute amounts of calcium and phosphorus in the diet of rats to the production of rickets, have been checked against the classic papers in the literature. Without undue citation it may be stated that, with no important exception, the diets fall in the fields as graphed. Thus a number of cases which when considered from the viewpoint of ratio alone seemed contrary to the expected result now fall into the larger and more general scheme. The relation of diet to the production of rickets has thus been clarified.

It is at once evident from histological and x-ray studies in this and the companion study (Brown et al., '32) that rickets may be either present or absent with almost any blood picture. Variations in the serum calcium and phosphorus can be interpreted as a quantitative measure of rickets only when the diet is fixed, as in vitamin testing; or in the rickets of infants, where milk determines the calcium and phosphorus intake. In our experiments the serum calcium values of 3.6 and 3.9 mg. per cent (less than 1 mM.) are as low and the phosphates, above 15 mg. (5 mM.) are as high as we have encountered in dietetic studies. The protein values in scattered analyses are roughly constant at 5.5 per cent and hence the fraction bound by protein is normal and constant. Such material would be especially suited for a study of the forms of calcium in the blood serum by the methods recently published by McLean and Hastings ('35). The ionized and other fractions could then be determined under conditions where the phosphate is the main variable.

The bone ash gives an excellent quantitative insight into the amount of mineral deposition but obviously cannot differentiate between rickets and osteoporosis, between failure to ossify or demineralization. In these studies values below 40 per cent (except at dietary ratio of 1.0, or with very high phosphorus intake) have been associated with rickets. Properly used this gives an excellent insight into the degree of ossification.

From a correlation of the above criteria it is clear that rickets in rats bears a closer relation to the found in infants and dogs than was formely supposed, in that rickets is produced with diets in which the ratio has been considered 'normal,' provided the amounts of calcium and phosphorus are sufficiently low. The essentials for the production of rickets are an inadequacy of vitamin D accompanied by relative deficiency of calcium or phosphorus or an absolute deficiency of either or of both.

Tetany is found associated with low calcium and high phosphate in the serum. Although such blood values were present in the animals made ricketic on high phosphorus low calcium diets, they showed only moderate tetany. But the same blood picture is present when animals with low phosphorus rickets or even normals are changed to high phosphorus diets. These animals respond with tetany of such violence as often to be fatal. This is presumably due to the rapid change in the blood from low phosphate to high phosphate with the rapid deposition of calcium phosphate in the bones.

In the present experiments the highest phosphate diets were not associated with the most marked tetany. The increased acidity of these diets may have been a factor, for it is well known that acid tends to prevent tetany and alkali to produce it. Salvesen, Hastings and McIntosh ('24) reported that when the ingested phosphate was moderate, the neutral salts produced greater effect than the acid or alkaline ones (compare also Shohl et al., '28 a). With larger doses, however, all three produced violent tetany. It is difficult to compare the size of a 50 mg. dose for a 50 gm. rat with a 5.0 to

7.0 gm. dose for a 13 to 20 kg. dog, and undoubtedly both the level of intake and acidity must be carefully evaluated.

In this experiment the principal interest lay in the analysis of rickets and not of tetany, and no further studies in acid-base were carried out. Previous experiments had shown that when severe tetany was present the rats could not or would not eat the food. Under these conditions rickets heals—therefore severe tetany was to be avoided as a complicating factor. The acid-base value of the diet has been shown previously to have a definite effect on the ricketogenic properties of the diet; the acid diets tend toward the production of rickets and the alkaline toward its prevention (Shohl et al., '28 a). In the type of diet used in this experiment the effect is secondary to the calcium and phosphorus relations (Shohl et al., '32). Preliminary experiments had shown that high phosphate diets, when neutral, were not ricketogenic (Brown, '30). In the present study no effort was made to keep the acidity constant throughout, but the acidity was permitted to increase with the KH_2PO_4 additions. This would tend toward increase in the amount of rickets produced, at a given ratio, as the levels increased; but the data show the converse, and hence the conclusion that high salt diets prevent rickets is further enhanced.

CONCLUSIONS

1. Serum calcium and inorganic phosphorus reflect the ratios of the diet. Rickets may be present when the serum phosphate or calcium or both are low. When the calcium is low and the phosphate high, tetany occurs. As the levels in the diet increase, at a given ratio, both serum calcium and phosphate and the per cent of ash of the fat-free bones increase, and rickets and tetany diminish. X-ray, and as a final criterion, the histology of the bones are necessary to form a judgment as to the presence or absence of rickets.

2. From a survey of the whole field of calcium and phosphorus relationship in the diet, in the absence of vitamin D, it is clear that rickets may be produced with not only high

calcium low phosphorus diets, and low calcium high phosphorus diets, but also with low calcium low phosphorus diets. The last group, not previously described, occurs in a zone in which the ratios of Ca/P have been called 'normal,' a designation which has lost its significance, for rickets may be produced with any ratio of Ca/P in the diet. As the absolute amounts are increased, for any given ratio, the diet changes from a ricketogenic to a non-ricketogenic diet.

I wish to express my thanks to Doctor Vogt of the x-ray department for the excellent roentgenograms and aid in their interpretation, and to Miss G. M. Rourke, Miss H. M. Blatt, and Miss L. F. Wright for technical assistance.

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THE HEALING OF RICKETS IN RATS ON A DIET CONTAINING NEGLIGIBLE AMOUNTS OF CALCIUM AND VITAMIN D [1]

JAMES H. JONES AND BERNARD N. E. COHN [2]

Department of Physiological Chemistry and Laboratory of Orthopedic Research, School of Medicine, University of Pennsylvania, Philadelphia

ONE PLATE (TEN FIGURES)

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While studying the influence of large doses of irradiated ergosterol on various tissues of young rats, György ('30) observed a marked decalcification of the long bones. The area most affected was that adjacent to the actively proliferating area which the author termed the subepiphyseal spongiosa. The provisional zone of calcification showed a sharp contour, a narrow epiphyseal line and no signs of proliferation of new bone. The diet used was low in calcium. It would appear that under the influence of large doses of irradiated ergosterol calcium salts had been deposited in the growing portion of the bone, while they had been withdrawn simultaneously from the ossified part. Jones and Robson ('33) also using a diet low in calcium, obtained somewhat similar results. They found that young rats which were maintained on the Steenbock-Black rachitogenic diet ('25) from which the $CaCO_3$ had been omitted developed a rachitic-like condition of the bones. The wide uncalcified area at the metaphysis, which is typical of rickets, was not prominent, but

[1] This investigation was aided by a grant made by the Faculty Research Committee of the University of Pennsylvania. A preliminary report of this work was read before the American Institute of Nutrition meeting at Detroit on April 10, 1935.

[2] Work done while a Fellow in Orthopedic Surgery, Graduate School of Medicine.

histological studies showed a marked overgrowth of osseous tissue at the ends of the long bones. Similar results were obtained on a diet composed solely of ground whole yellow corn to which was added 1 per cent sodium chloride. Although these diets contained very little calcium and were low in phosphorus, the addition of irradiated ergosterol prevented the overproduction of osseous tissue, and bones of apparently normal architecture were obtained. The cortex, however, and spongiosal trabeculae were very thin and poorly calcified. Chemical analyses indicated that the absolute amount of ash in the femora of the animals receiving the vitamin D supplement was no greater than in the femora of the rachitic animals. Since there had been deposition of calcium salts in the metaphyseal region without increase in total bone ash there must have been withdrawal of salts from another part of the bone. Venar and Todd ('34) obtained results very similar to those of Jones and Robson.

If a deposition of calcium salts in the actively proliferating area of the bone can be coincident with their absorption from the shaft it is possible that rickets could be cured on a diet devoid of calcium. Such a study has been made in the following experiments in which were determined the effects of feeding a ration containing at most only traces of calcium to rats previously made rachitic on a modified Steenbock-Black diet (Jones, '34). The low calcium diet was fed with and without the addition of vitamin D.

EXPERIMENTAL

Several variations in the low calcium diet were used in an attempt to get a diet very low in calcium and vitamin D but otherwise adequate for growth. It was hoped that a diet sufficiently low in calcium could be obtained so that the ash from 10 gm. would not give a positive test for this element by the oxalate method. The diet finally adopted is shown in table 1.

The casein was made calcium free by the following procedure: 500 gm. of casein were allowed to stand overnight in

10 liters of approximately 0.1 N HCl. The supernatant acid solution was then removed and the casein dissolved in dilute NaOH and re-precipitated with acetic acid, washed with distilled water and again allowed to stand overnight in 0.1 N HCl. This process was repeated for 10 to 14 days; then the casein was washed and dried. Due to the possible destruction of the sulfur-containing amino acids during this treatment, as reported by Jones and Gersdorff ('34), a small amount of cystine was added. The salt mixture used is a modification of the Steenbock salt no. 40 ('23) from which the calcium phosphate and lactate were omitted and an equivalent amount of phosphorus as disodium phosphate was added. The salts

TABLE 1

Composition of low calcium diet

	per cent
Casein (acid and alkali treated)	14.00
Cystine	0.05
Straw (acid washed)	4.00
Salt mixture[1]	4.00
Lard	20.00
Lactose	15.00
Sucrose	22.45
Sucrose + wheat embryo extract	20.00
Liver extract	0.40
Carotene solution	0.10

[1] Steenbock salt no. 40 from which the calcium phosphate and lactate were omitted and an equivalent amount of phosphorus as disodium phosphate was added.

used were C.P. except the ferric citrate which was U.S.P. Roughage was furnished by 4 per cent of rye straw which was freed from calcium by washing with 0.1 N HCl. After drying, it was finely ground in a ball mill. Energy was provided by a combination of lard, sucrose and C.P. lactose. Vitamin A was supplied as an impure solution of carotene and the vitamin B factors were furnished by an 80 per cent alcoholic extract of wheat embryo and a small amount of liver extract.[3] The wheat embryo extract was evaporated on sucrose and made up to a weight equivalent to the original weight of embryo.

[3] Eli Lilly and Co. no. 343.

Several samples of this diet gave no test for calcium while in others a maximum of 0.3 mg. per 10 gm. of diet was obtained. The animals, therefore, received at most about 0.2 mg. of calcium per day as they consumed from 3 to 7 gm. of the ration. The diet contained 0.46 per cent phosphorus. Metabolism experiments showed that the animals were in a state of decidedly negative calcium balance. Individual animals were placed in cages and feces and urine collections were made for periods of 1 week. The excreta as well as the diet were analyzed for calcium. Table 2 gives the results of a few such experiments. The amount of calcium lost varied considerably with individual animals but the total calcium excreted was always greater than the intake.

TABLE 2

Calcium balances of rats on low calcium diet

RAT NO.	PERIOD [1]	DIET	CALCIUM		
			Intake [2]	Output	Loss
				mg.	*mg.*
1	1st	Low calcium + irradiated ergosterol	0.93	5.81	4.88
1	2nd	Low calcium + irradiated ergosterol	1.08	9.74	8.66
2	1st	Low calcium + irradiated ergosterol	0.90	14.55	13.65
2	2nd	Low calcium + irradiated ergosterol	0.75	28.63	27.88
3	1st	Low calcium	0.57	2.42	1.85
4	1st	Low calcium	0.78	4.68	3.90

[1] Collections made for periods of 7 days each.

[2] Calculated on basis of analysis of diet giving highest value for calcium.

Almost immediately after the rachitic animals had been transferred to this diet their intake of food became greatly reduced and several developed tetany in less than 20 hours after the change. Within 2 days nearly all the animals showed signs of tetany and a few deaths occurred. Up to this point the results were very similar to those obtained when rachitic rats were fasted. Forty-eight hours after the transference to the low calcium ration the animals were divided into three groups: The animals of group I were killed immediately by bleeding; group II was continued on the low calcium diet, and group III was given in addition 200 international units (I.U.) of vitamin D per day in the form of

irradiated ergosterol.[4] Following the 48-hour period the food consumption of groups II and III increased; signs of tetany abated or completely disappeared, and most of the animals gained weight. The death rate of the animals of group II was considerably higher than that of group III. The animals of the latter group were sacrificed at weekly or bi-weekly intervals for a period of 8 weeks. From two to four were killed at a time. Owing to the high death rate in group II it was impossible to sacrifice these animals at given intervals,

TABLE 3

Representative determinations of femur ash and serum calcium and phosphorus of rats on low calcium diet

LOT NO.	NUMBER OF ANIMALS	DAYS ON DIET	IRRADIATED ERGOSTEROL I.U.	FEMUR ASH [1]		SERUM [2]	
				Milligrams	Per cent	Ca	P
1	3	2	0	24.2	31.4	6.2	10.5
2	3	2	0	24.3	32.3	6.7	10.9
3	3	23	0	22.8	29.0	5.4	12.5
4	2	29	0	33.4	31.9	9.6	7.0
5	4	37	0	29.6	36.8	7.6	10.0
6	3	9	200	25.5	36.6	7.8	13.7
7	3	23	200	26.2	35.1	9.0	9.2
8	3	44	200	26.5	32.1	10.5	11.1
9	4	58	200	31.9	28.1	9.2	9.8

[1] Average of lot.
[2] Analysis of pooled sera.

but several lived long enough to give definite results. The blood sera were pooled and analyzed for calcium by the method of Clark and Collip ('25) and for phosphorus on the calcium-free filtrate by the method of Gunther and Greenberg ('29). The right femur of each animal was removed for ash analysis; the wrists were taken for examination by the line test technic, and the remainder of the skeleton was preserved for histological study.

In table 3 are shown the results of some of the chemical analyses which are typical of the entire series. The first two

[4] Furnished through the courtesy of Mead Johnson and Co., Evansville, Ind.

lots of animals were of group I and were killed after 48 hours on the low calcium diet. It can be observed that the calcium of the serum was low and the phosphorus was high. The next three lots were of group II and were continued on the low calcium diet for approximately 3, 4 and 5 weeks, respectively. At the end of 3 weeks the serum calcium was still low, but there was a slight increase by the fourth and fifth weeks. The phosphorus of the serum for the most part remained high. The next four lots were of group III and received irradiated ergosterol for periods of 1, 3, 6 and 8 weeks. The time in days as given in the table included the 2 days during which these animals did not receive the irradiated ergosterol. At the end of 1 week the serum calcium was only a little above that of the animals of group I. However,

TABLE 4

Average ash content of femora of all animals of each group

DIETARY GROUP	NUMBER OF ANIMALS	FEMUR ASH	
		Milligrams	Per cent
I. 48 hours on low calcium diet	20	24.5	31.5
II. Continued without irradiated ergosterol	20	28.0	33.3
III. Continued with irradiated ergosterol	19	28.6	33.7

the animals which received vitamin D for a longer period of time had a calcium level but slightly below normal. The phosphorus was more variable than the calcium and, on the whole, tended to remain high, attaining in a few cases the extremely high levels of 15 to 20 mg. per 100 cc. of serum.

The ash analyses of the femora showed slight variations. There appeared to be a general tendency toward an increase in the absolute amount of ash as well as in the percentage of ash in the femora of the animals of groups II and III as compared to group I. The increases in the ash are shown best by the averages of a larger number of animals which are given in table 4. The differences are slight but consistent, and considering the number of animals used are probably significant. No increase in the absolute amount of ash was

observed until the animals had been on the low calcium diet for at least 4 weeks. If this apparent increase in absolute amount of ash is real, the gain must have been at the expense of the ash content of other parts of the skeleton because as previously stated the animals were in a negative calcium balance. The increase in percentage of ash, on the other hand, might have been due to an actual decrease in organic matter as observed by Jones and Robson ('33).

Plate 1 shows the results of a few representative line tests on the wrists. The first photograph is that of a typical rachitic bone while the next five are of bones taken from animals which had been on the low calcium diet for 48 hours. It will be observed that there is considerable individual variation in the degree of healing[5] at this time. In the first two there is no sign of healing, in the next there is a slight but continuous line, and in the last two the degree of healing is still greater. The next two photographs are of bones taken from animals which received irradiated ergosterol for 5 weeks. In these instances the healing is complete as shown by the narrow regular epiphyseal line. Moreover, the rachitic enlargement of the end of the bone has decreased and the architecture of the bone appears normal. The extent of calcification, however, is slight as shown by the thinness of the cortex. The last two photographs are of bones taken from animals that were fed the low calcium diet without irradiated ergosterol for 30 and 57 days, respectively. In the former healing is complete as judged by the degree of calcification. However, the architecture is not entirely normal as shown by the presence of a large amount of cancellous bone and the persistence of the enlargement at the end of the diaphysis. On the other hand, the metaphysis of the radius of the rat which was on the low calcium diet for 57 days is not only completely calcified, but also appears to be as normal as those from animals which received vitamin D. Here again the cortex is extremely thin, and the entire bone shows a deficiency of calcium salts. Detailed histological studies of the

[5] As used in this paper the term healing means the degree of calcification at the rachitic metaphysis.

bones and other tissues of these animals will be reported at a later date.

From these data it appears that in the rat on a diet deficient in calcium but adequate in phosphorus, calcium salts can be transferred from one part of the skeleton to another. That is, there can be a simultaneous withdrawal of bone salts from one part of the skeleton and a deposition of these salts in another part. This transfer can be of sufficient magnitude to produce healing of rickets in rats in a few weeks and the antirachitic factor is not essential for this process. Vitamin D, however, apparently decreases the time necessary for the healing to take place. It also tends to protect the animal against the calcium deficiency as shown by the level of calcium in the blood serum and in the mortality rate of the animals on the low calcium diet.

SUMMARY

Young rachitic rats, which were transferred to a synthetic diet of adequate phosphorus content but containing at most only traces of either calcium or vitamin D, showed a marked decrease in food consumption and developed tetany within 18 to 48 hours. After 48 hours the animals were divided into three groups: group I, sacrificed; group II, continued on synthetic diet; group III, given in addition irradiated ergosterol. Serum calcium and phosphorus of group I were approximately 6 and 10 mg., respectively, and the line test indicated slight healing. The food consumption of groups II and III increased after 48 hours and tetany disappeared. Serum calcium increased while phosphorus remained high. After a few weeks both groups of animals showed complete healing of the rickets. The absolute amount of ash in the femora of the animals of groups II and III appeared to be slightly more than those of group I. Since healing of the rachitic lesions and the possible slight increase in femur ash occurred on a diet containing negligible amounts of calcium the bone salts must have been transferred from calcified portions of the skeleton to the more rachitic parts. Furthermore,

as healing took place in the animals not receiving irradiated ergosterol vitamin D is apparently not essential for this transference.

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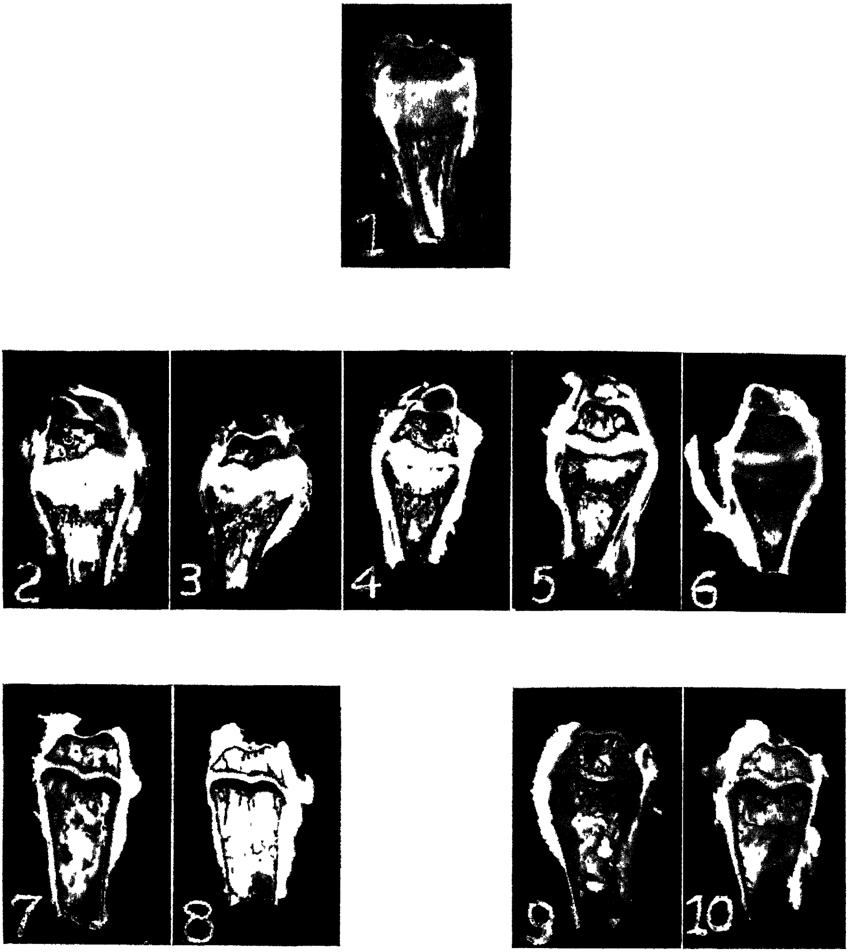

Healing of rickets in the radii of rats on a diet containing only traces of calcium.

1 Rachitic control.
2 to 6 Forty-eight hours on calcium-free diet without irradiated ergosterol.
7 to 8 Thirty-five days on calcium-free diet with irradiated ergosterol after first 2 days.
9 Thirty days on calcium-free diet without irradiated ergosterol.
10 Fifty-seven days on calcium-free diet without irradiated ergosterol.

THE EFFECT OF QUANTITATIVE UNDERFEEDING AND OF VITAMIN A DEFICIENCY ON THE TISSUE LIPIDS OF RATS FED DIETS LOW IN CHOLESTEROL

HELEN L. GILLUM AND RUTH OKEY[1]
Laboratory of Household Science, University of California, Berkeley

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Studies of the effect of undernutrition not only on the total quantity but also on the percentage distribution of the tissue lipids (Meyer and Schaeffer, '14; Terroine, '14) have been responsible to some degree for our concept of lecithin and cholesterol as active constituents of tissue which take part in the essential life processes of the cell, and persist even in prolonged inanition and after the supply of storage or depot fat has been exhausted.

The literature on the effect of vitamin A deficiency on tissue lipids is somewhat contradictory. Some of the papers dealing with fat deposition in young animals suggest that the effect noted may be due entirely to undernutrition while others indicate that vitamin A may exert a specific influence on tissue lipid function and distribution.

Much of the classic work on the effect of inanition on tissue lipids was carried out before the development of methods for chemical estimation of the individual lipid constituents of the tissue, or of present day technique for the feeding of animals on adequate diets made up of purified food stuffs.

[1] The material presented in this paper is taken from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree in household science in the graduate division of the University of California, Berkeley, May, 1935.

A parallel investigation of the effect on tissue lipids of 1) a limited food intake with adequate vitamin supplements and of 2) a vitamin A deficient diet, therefore appeared to be indicated. As a control it was felt that the investigation should first be carried out with diets which would give as nearly as possible 'endogenous' conditions of lipid metabolism.

We have therefore carried out:

1. A preliminary investigation of the lipid content of the easily separable tissues from the carcasses of a series of a) A deficient rats (FA–); b) litter mate controls on limited food intake, but with adequate vitamin supplements (FU); c) litter mate controls fed an adequate diet ad libitum (FN).

2. A more detailed study of the liver lipids of animals in the same three states of nutrition.

Data from this latter investigation is presented in the following paper of the series.

The basal diet used was:

Casein—baked and alcohol ether extracted	18
Crisco—(irradiated to supply 'D')	5
Agar	2
Osborne Mendel salt mixture	4
Corn starch	71

The vitamin supplements were:

A. Cod liver oil (Squibbs standardized). For the control and undernourished groups, 1 drop daily until the rats were 8 weeks of age and 2 drops thereafter; for the A deficient animals, the same amounts of oil heated to 100°C. until its A content, as determined by feeding tests, had been destroyed.

B. Dried brewer's yeast. Given as separate supplements to all groups to the extent of 4 per cent of the average food consumption until within 3 weeks of killing when 100 mg. of Harris yeast extract was substituted to guard against the immediate effects of feeding excess yeast sterols.

It should be noted that apart from the vitamin supplements this diet was sufficiently nearly sterol free to give a negative Liebermann-Burchard reaction when the alcohol ether soluble material from 1 gm. of diet was extracted with chloroform.

The lecithin content was limited to that in the yeast and cod liver oil, and the fat content held relatively low.

Grouping of experimental animals

Litters were reduced to nine, six males and three females or three males and six females, as soon as sex could be determined. Animals of the same litter and sex were divided into three groups designated as:

1. A deficient (FA), given the basal diet ad libitum with the standard yeast supplement but with heated cod liver oil.
2. Undernourished (FU), limited to an intake of basal diet which permitted only the weight gains made by the A deficient groups, but given adequate vitamin supplements.
3. Controls (FN), given the basal diets ad libitum with adequate vitamin supplements.

Plan of feeding

All animals used were placed on the basal experimental diet at weaning. The food supply of the 'undernourished' group was determined by actual weighing of the food consumed by the A. deficient partner.

Control animals of all groups were killed at the same time as the A deficient animals, i.e., after about 70 days on the experimental diet, or when the A deficient animals showed rather serious avitaminosis.

The procedures used for killing the animals and handling the tissues have been described in a previous paper (Okey, Gillum and Yokela, '34). Except as noted below the analyses reported in the tables were made by micro oxidation procedures (Bloor, '28, '29; Okey, '30). In most cases the total cholesterol determinations were also checked by a colorimetric procedure (Bloor, Pelkan and Allen, '22), and in table 1 the figures for total cholesterol reported are from colorimetric estimation.

Data from the preliminary study of pooled samples of the various tissues are given in table 1. (Data from a larger series of liver lipid analyses are given as controls in the next paper.)

TABLE 1

Lipid constituents of tissues. Preliminary series

GROUP		NUMBER OF ANIMALS	FATTY ACID	TOTAL STEROL[1] PER CENT MOIST TISSUE	LECITHIN PER CENT MOIST TISSUE	MOISTURE PER CENT
Liver						
FA-5		4	4.62	0.27	2.27	69.9
FU 5		4	3.25	0.27	2.70	70.7
FN 5		4	3.25	0.25	2.76	70.8
FA-6		4	7.47	0.32	2.44	
FU 6		4	6.38	0.40	3.47	
FN 6		4	7.45	0.32	2.54	
Hearts						
FA-5	A-6	8	1.98	0.14	1.89	73.9
FU 5	U 6	8	2.16	0.12	1.98	75.8
FN 5	N 6	8	2.26	0.13	2.04	77.4
Kidneys						
FA-5		4	5.32	0.47	1.89	70.2
FU 5		4	5.47	0.54	2.53	73.3
FN 5		4	Extract lost			71.2
FA-6		4	4.49	0.52	2.07	
FU 6		4	4.83	0.59	1.79	
FN 6		4	5.56	0.52	2.11	
Lungs						
FA-5	A-6	8	3.67	0.46	1.65	75.2
FU 5	U 6	8	3.34	0.49	2.04	78.4
FN 5	N 6	8	3.72	0.45	1.99	77.9
Spleen						
FA-5	A-6	8	2.32	0.35	1.40	73.2
FU 5	U 6	8	2.72	0.35	1.54	74.9
FN 5	N 6	8	2.66	0.33	1.54	76.3
Brain						
FA-5	A-6	8	3.44	1.28	3.55	78.7
FU 5	U 6	8	3.53	1.16	3.28	78.6
FN 5	N 6	8	3.67	1.28	3.89	79.1
Muscle						
FA-6		4	3.59	0.10	0.92	Lost
FU 6		4	6.40	0.10	0.87	74.0
FN 6		4	4.10	0.09	0.82	72.8
Skins						
FA-5		4	16.9	0.25	0.55	52.5
FU 5		4	15.3	0.26	0.54	54.2
FN 5		4	26.2	0.24	0.50	45.8
FA-6		4	17.9	0.29	0.63	
FU 6		4	21.2	0.26	0.62	
FN 6		4	25.0	0.19	0.62	
Residue						
FA-5		4	5.75	0.15	0.99	71.7
FU 5		4	4.44	0.15	1.07	68.5
FN 5		4	9.90	0.12	1.04	65.0
FA-6		4	6.48	0.14	1.12	
FU 6		4	5.23	0.13	1.08	
FN 6		4	7.50	0.11	1.14	

[1] Total sterol was determined colorimetrically in all cases except the liver, in which case the digitonide method was used.

The comparatively small differences observed in the total lipid of moist tissues of the different groups may probably be taken as a check of technique in freeing the samples from adherent body fat. From the figures for 'ground residue' which included a large proportion of bone, muscle, connective and adipose tissue, it will be seen that the 'undernourished' animals usually had even less body fat than the A deficient animals. Likewise the effect of undernutrition or vitamin deficiency was very evident in the total lipids of the skin.

Cholesterol and lecithin values show only to a very slight degree the higher proportionate retention which might be expected in the undernourished animals. That the moisture content of a number of tissues varied, usually in the direction of higher values in the control than in the vitamin deficient animals, may possibly be indicative of differences in rate of metabolism, i.e., if we may apply the hypothesis that rapidly metabolizing cells will have a higher water content than older and less active ones.

It must be emphasized that all of the data in table 1 are from the analysis of tissues of rats fed on an almost sterol and lecithin free diet, containing only 5 per cent fat and therefore may probably be taken to indicate nearly minimum variations in tissue lipids. Hence it is perhaps not strange that on the basis of Bloor's hypothesis that the lecithin/free cholesterol ratio in a tissue may be taken as an index of its metabolic activity (Bloor, Okey and Corner, '30), we found relatively little evidence of decreased cellular activity in the A deficient and undernourished animals. As a basis for distinguishing between differences in lipid content of tissues inherent in, or directly dependent upon, general condition of nutrition and not exaggerated by specifically stimulating factors in the diet, these data have appealed to us as important.

SUMMARY AND CONCLUSIONS

Data are presented from analyses of the readily separable tissues of twenty-four rats on a low sterol basal diet in the following three states of nutrition, a) vitamin A deficient, b) limited as to food intake but given adequate vitamin supplements, c) normal controls.

The percentages of lipid constituents found varied rather markedly from tissue to tissue, but apart from neutral fat, the differences observed in the lipid constituents of the individual tissues when considered with relation to the nutritional state of the animals were slight, occasionally inconsistent, but generally in the direction to be expected from earlier work on tissue lipids in undernutrition. There was no clear evidence of a specific effect of A deficiency.

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THE EFFECT OF QUANTITATIVE UNDERFEEDING AND OF VITAMIN A DEFICIENCY ON THE LIVER LIPIDS OF RATS FED DIETS WITH ADDED CHOLESTEROL

HELEN L. GILLUM AND RUTH OKEY[1]
Laboratory of Household Science, University of California, Berkeley

THREE FIGURES

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In the preceding paper of this series (Gillum and Okey, '36) we reported a study of the lipid constituents of the tissues of rats, a) quantitatively underfed, b) vitamin A deficient, and c) controls. All were given a basal diet of low lecithin, cholesterol and fat content designed to give a picture of 'endogenous' tissue lipid variations in the three states of nutrition.

The present paper deals with a similar study of variations in the lipid content of tissue when one lipid constituent of the diet is increased to the point of producing a marked tissue change even in control animals. Because of the known influence of cholesterol feeding on liver lipids, and of the striking change in the liver lipids observed by one of us when cholesterol fed rats were deprived of vitamin B, study of the liver lipids of cholesterol fed rats in the three states of nutrition seemed best adapted to our purpose.

The basal diet and the plan of feeding was the same as that reported in the preceding paper, except that for approximately half of the rats from each group, 1 per cent of

[1] The data in this paper are taken from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree in household science in the graduate division of the University of California, Berkeley, May, 1935.

A preliminary report of some of this work was made at the meeting of the American Society of Biological Chemists, Detroit, April, 1935.

cholesterol was incorporated into the basal diet when the rats were about 6 weeks of age.

This meant that the animals were divided into six groups as follows:

1. FA, A deficient animals on sterol low diets.
2. CA, A deficient animals on 1 per cent cholesterol.
3. FU, quantitatively underfed animals (with adequate vitamin supplements) on sterol low diets.
4. CU, quantitatively underfed animals on 1 per cent cholesterol.
5. FN, control animals (fed the sterol low diet ad libitum with adequate vitamin supplements).
6. CN, control animals (fed the 1 per cent cholesterol diet ad libitum with adequate vitamin supplements).

The cholesterol fed animals deprived of vitamin A in the form of cod liver oil developed the typical signs of vitamin deficiency at about the same time as their partners on the sterol low diet. They, with their undernourished and normal controls, were killed after approximately 50 days of cholesterol feeding or at about 90 days of age.

The procedure for killing the rats and for making and analyzing the tissue extracts was exactly as described in the previous paper except that samples were taken from individual animals rather than pooled tissues from several animals of the same group.

Figures for total cholesterol are, however, in all cases quoted from the results of the oxidation of the digitonid rather than the colorimetric estimation. Average figures for the two sexes in the different groups of the second series of animals are reported in table 1.

Graphic comparison of all the rats as a whole are given in figures 1, 2 and 3.

It will be seen that, in general, the differences in composition between the livers of the control and the A deficient and 'undernourished' groups tended to become exaggerated when the animals were given cholesterol.

TABLE 1

A comparison of the averages of the liver lipids of vitamin A deficient, undernourished and control rats on a 5 per cent fat diet containing no added cholesterol, with those of similar animals on the same diet containing 1 per cent added cholesterol, tabulated by sex

Diet	FA−		FU		FN		CA−		CU		CN	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Number of animals	5	5	6	2	5	6	6	5	6	5	6	5
Weight of rat grams	140	103	136	125	214	165	150	135	147	132	231	180
Moist weight liver grams	4.48	2.98	4.41	2.78	6.14	4.75	5.43	4.42	5.51	4.76	8.01	6.52
Dry weight liver grams	1.40	0.88	1.39	0.81	1.94	1.58	2.03	1.84	2.11	1.79	3.27	2.73
Moisture per cent	68.6	70.1	68.8	71.0	68.3	66.8	63.0	60.0	61.8	60.3	59.2	58.1
Fatty acids per cent moist weight	6.6	4.7	4.0	2.9	5.4	9.9	10.5	13.1	10.7	14.2	15.7	14.7
Fatty acids per cent dry weight	20.6	13.0	12.6	10.0	16.9	29.4	28.7	30.8	27.8	32.8	38.7	35.1
Fatty acid milligrams per rat	290	141	188	81	328	470	606	577	602	691	1271	966
Fatty acids milligrams per 100 gm.	206	134	133	64	153	285	417	436	397	513	548	535
Total cholesterol per cent moist weight	0.34	0.33	0.31	0.24	0.33	0.30	4.5	4.9	7.0	5.6	5.5	4.7
Total cholesterol per cent dry weight	1.08	1.07	0.98	0.83	1.06	0.92	12.4	12.4	18.5	13.1	13.4	11.2
Total cholesterol milligrams per rat	15.1	9.9	14.4	6.7	20.4	14.5	263	216	389	272	443	314
Total cholesterol milligrams per 100 gm. rat	10.8	9.6	10.3	5.2	9.5	8.7	180	166	264	202	194	174
Free cholesterol per cent moist weight	0.22	0.23	0.27	0.29	0.25	0.25	0.39	0.39	0.40	0.38	0.37	0.32
Ester cholesterol per cent moist weight	0.12	0.10	0.05	0.00	0.08	0.05	4.1	4.5	6.6	5.2	5.1	4.4
Lecithin per cent moist weight	2.8	3.0	2.9	2.1	3.1	2.9	2.4	2.7	2.8	2.7	2.8	2.8
Lecithin per cent dry weight	9.1	10.8	9.1	7.3	9.8	8.6	6.8	6.9	7.3	6.9	6.8	6.6
Lecithin milligrams per rat	127	90	135	59	189	137	133	121	148	129	220	180
Lecithin milligrams per 100 gm. rat	91	89	96	47	89	83	94	92	101	98	96	100
Lec./total cholesterol	8.5	9.2	9.2	9.0	8.9	9.6	0.63	0.61	0.40	0.53	0.54	0.62
Lec./free cholesterol	13.4	14.0	10.7	7.1	12.1	11.5	6.3	6.9	7.1	7.5	7.4	8.6

The higher moisture content of the livers of the animals on the poorer diets may probably be correlated with the lower fat and cholesterol content.

The reversal of difference between the fatty acid content of the livers of the undernourished and A deficient animals on the two basal diets may probably be explained by the very high ester cholesterol retention in the CU group. When there

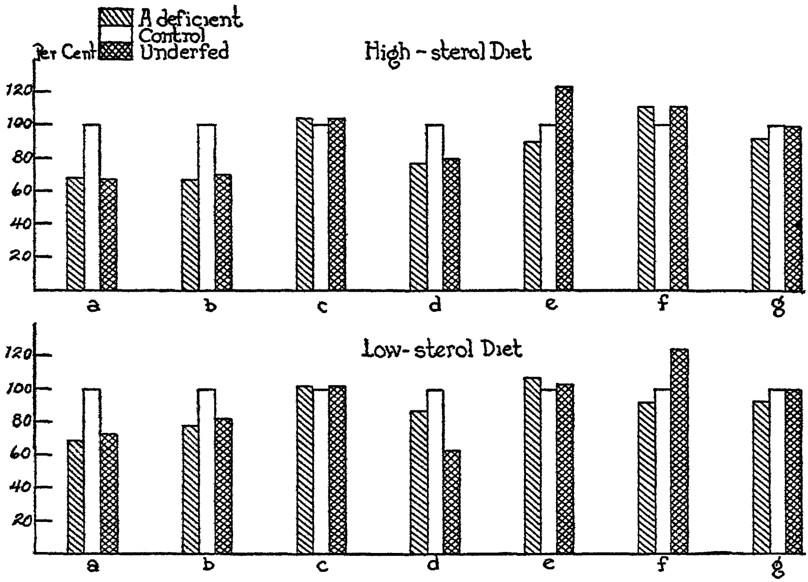

Fig. 1 A comparison of the liver constituents of vitamin A deficient, 'undernourished,' and control rats, on the basis of moist weights. The controls are taken as 100 per cent. a, weight of rat; b, moist weight of liver; c, moisture per cent; d, fatty acids, per cent moist weight; e, total cholesterol, per cent moist weight; f, free cholesterol, per cent moist weight; g, lecithin, per cent moist weight.

is no excess cholesterol in the diet, the 'undernourished' animals apparently lose an even greater proportion of fatty acid than the A deficient animals, and both these groups have far less liver fatty acid than the normal controls.

Ester cholesterol percentages for the cholesterol fed rats were, strangely enough, higher for the 'undernourished' than the normal controls. This meant not only a higher concentration (average 6.2 per cent) but almost as high an absolute

amount (average 336 mg.) in the relatively small livers of the undernourished group as in the greatly enlarged livers of the cholesterol fed controls (average 5.2 per cent and 385 mg.), and while there was a somewhat lower concentration (average 4.3 per cent) and a lower absolute amount (average 174 mg.) in the livers of the A deficient group CA, there was no such profound decrease from the control levels as that found by Okey ('33) for vitamin B deficient rats.

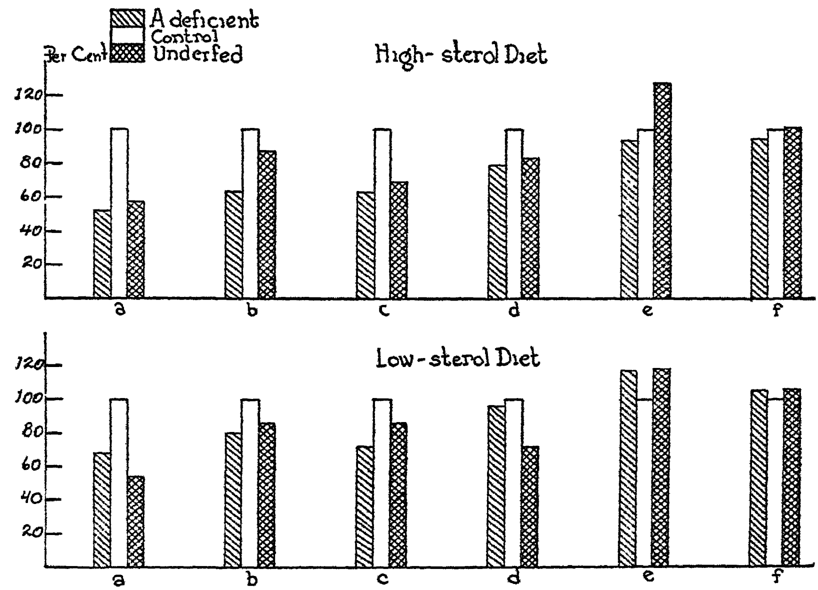

Fig. 2 A comparison of the liver constituents of vitamin A deficient, 'undernourished,' and control rats, on the basis of weight. The controls are taken as 100 per cent. a, fatty acids, milligrams per rat; b, total cholesterol, milligrams per rat; c, lecithin, milligrams per rat; d, fatty acids, milligrams per 100 gm. rat; e, total cholesterol, milligrams per 100 gm. rat; f, lecithin, milligrams per 100 gm. rat.

That there is a marked effect upon sterol storage which is associated with a condition of undernutrition appears to be evident. The actual total intake of cholesterol of the undernourished group was only slightly less (4.6 gm.) than that of the vitamin deficient animals (5.1 gm.), but was only about half that of the controls (CN group) (7.0 gm.). For the

last 2 weeks before killing the CA group averaged 1.1 gm., the CU group 1.0 gm., and the CN group 1.9 gm.

The fact that the underfed rat supplied with adequate vitamin supplements had an actual storage of 87 per cent of that of the controls with an intake only 53 per cent as great would seem to indicate some profound difference in the reaction of the two groups to the high sterol diet. It must be noted that all of these animals were on diets containing only 5 per cent

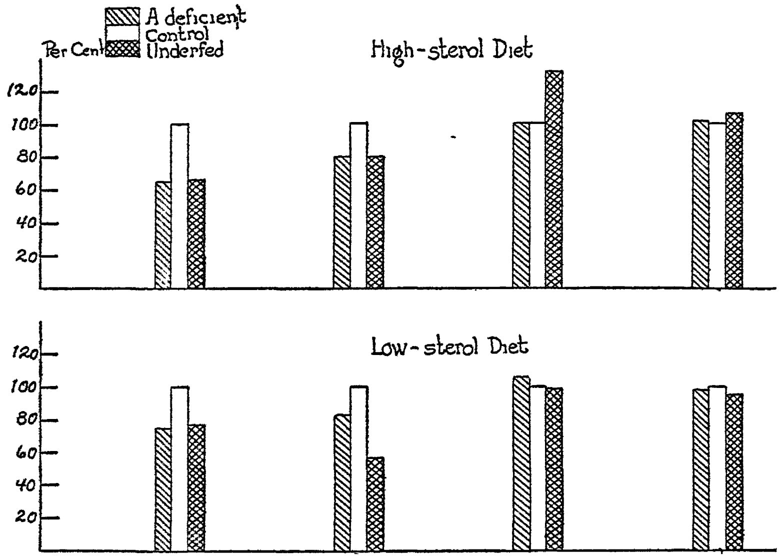

Fig. 3 A comparison of the liver constituents of vitamin A deficient, 'undernourished,' and control rats, on the basis of dry weights. The controls are taken as 100 per cent. a, dry weight of liver; b, fatty acids, per cent dry weight; c, total cholesterol, per cent dry weight; d, lecithin, per cent dry weight.

fat, and that even the normal controls stored less fat and less cholesterol ester than at higher levels of fat intake.

Cholesterol balance experiments were made with certain groups of the animals. They gave by colorimetric estimation some evidence of better cholesterol absorption in the control than in the A deficient groups. However, the dependability of colorimetric determinations of cholesterol in fecal extracts necessarily contaminated by material which interferes with the Liebermann-Burchard reaction is very doubtful. On the

other hand, many of the products of reduction of cholesterol as well as coprosterol are not quantitatively precipitated by digitonin. Hence it was felt that the reliability of methods available did not justify further work on this point.

The enlargement of the livers of the cholesterol fed controls, together with their tendency to store a great deal of cholesterol ester per rat, with a lower concentration per gram of tissue than in the undernourished animals, may be due to an inability of the liver of the latter to enlarge to meet the demands of metabolic emergency imposed by the high sterol diet. The situation in the A deficient animal suggests something of the same sort, partially ameliorated due to the poor absorption and utilization of food accompanying the vitamin deficiency.

Average lecithin concentrations in the moist livers were found to vary within a remarkably narrow range regardless of the basal diet or the state of nutrition of the animals. Analysis of individual variations observed within any group suggests that these may be partially accounted for by the unavoidable slight differences in time the rats were without food before killing and in food consumption during the day before food cups were removed from cages. The average of the entire A deficient group was 2.7 per cent which represents a decrease of 6.9 per cent from the values for the control group. This is in good agreement with the findings of Javillier et al. ('29); and of Monaghan ('32) who reports lowered phospholipids accompanying avitaminosis, and in fasting.

Phospholipid to cholesterol ratios

On the basis of the hypothesis of Bloor et al. ('30) that the lecithin/free cholesterol ratios may be taken as an index of metabolic activity, we can find no evidence of decreased cell activity in the vitamin A deficient or undernourished animals fed diet F. But when a diet containing cholesterol was ingested there was a marked decrease in the value of this ratio, and the proportionate decrease was greater in the case of the A deficient animals.

It is possible, therefore, to consider that cholesterol feeding decreases the proportion of actively metabolizing tissue in the liver, and that there is an exaggeration of this effect in the A deficient animals.

Effect of sex

It will be seen from the table that the differences between the liver lipids observed for the control animals of this series attributable to sex were in agreement with those previously reported from this laboratory (Okey, Gillum and Yokela, '34). That is, there was a higher concentration and a higher absolute amount of cholesterol in the livers of the males than of the females. That this difference became practically negligible in the case of the A deficient animals did not seem so strange in view of the sexual underdevelopment usually associated with deficiency in this vitamin. But that the differences between liver lipids in males and in females should be even greater in the undernourished groups than in the controls is somewhat difficult to explain in view of the relative infantilism of reproductive organs in the 'undernourished' animals at autopsy.

Unfortunately the pooled samples used for the analyses of liver lipids in the early groups included in this study were made up of tissues usually from two males and two females, and hence not separable by sex, and the later series did not contain enough animals to justify definite conclusions based on statistical comparison. Further investigation should prove interesting.

SUMMARY

Data are reported showing the effect of cholesterol feeding on the liver lipids of rats which were, a) vitamin A deficient, b) limited as to food intake but given adequate vitamin supplements, c) normal controls.

The size of the livers and their fatty acid content was less for the undernourished and A deficient animals both with and without cholesterol than for their respective controls.

Free cholesterol variations in liver were slight from group to group, but on both basal diets the 'undernourished' animals seemed to show a tendency to have a slightly higher concentration of free cholesterol than either of the other groups.

On diets low in cholesterol, lecithin and fat, the ester cholesterol concentration was low in the livers of all groups, and the variations of doubtful significance. When the rats were given cholesterol, the following changes were observed:

1. A lower concentration and a lower absolute amount of ester storage in the livers of vitamin A deficient than in either of the other groups. This was, however, of such a degree as to suggest poor assimilation rather than a large specific effect such as that observed in B deficiency.

2. A higher concentration and almost as high an absolute amount of cholesterol ester in the relatively small livers of the 'undernourished' animals as in the grossly enlarged livers of the controls.

3. A slightly lower phospholipid concentration was observed in the livers of the vitamin A deficient animals than in either of the other two groups on each basal diet.

The writers acknowledge their indebtedness to the board of research of the University of California for the funds which made this work possible.

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BASAL METABOLISM OF WYOMING UNIVERSITY WOMEN

ELIZABETH J. McKITTRICK[1]

University of Wyoming, College of Agriculture and Experiment Station, Laramie

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During the last few years the subject of basal metabolism has become increasingly important, and quite recently the basal metabolism of college women has claimed the attention of a number of educational institutions in various parts of the United States located at varying altitudes. Tilt ('30) published results of basal metabolism of young college women in Florida. Her subjects ranged from 17 to 25 years of age with the greater number falling in the 19- and 20-year groups. The altitude of Tallahassee, where the work was done, is 160 feet above sea level. Coons ('31) reported on the basal metabolism of Oklahoma women. Her subjects ranged from 17 to 36 years of age, with the greater number falling in the 19- to 25-year age groups. The altitude at Stillwater, where this report came from, is 870 feet above sea level.

Both of these studies reported metabolic rates well below the Aub Du Bois and Harris-Benedict standards, the usual standards used. McKay ('30) published a study on the basal metabolism of young women in Ohio. Her subjects ranged in age from 14 to 24 years, but all except five of the young women were from 14 to 18 years of age. The fact that so many of this group fell in the lower age groups makes this study less comparable with the other two, but here also all of the metabolic rates were below the Aub Du Bois standard, and the upper age group or 21 to 24 years was below the Harris-Benedict standard.

[1] The writer wishes to express appreciation to Dr. E. L. Sederlin for his interest and advice during this experiment.

Since the University of Wyoming is located at an altitude of 7148 feet and many people coming here to live have difficulty in adjusting themselves to the effects of the altitude, it seemed worth while to make a study of the basal metabolism of college women here to find out if the metabolic rate of college women was higher than at a lower altitude.

Another thing that added interest to the problem was the fact that a physician, who had practised in Ohio previously and had there, at an altitude of about 650 feet, felt the need of thyroid to speed up his metabolism, upon coming here to live was able to discontinue its use entirely.

EXPERIMENTAL SUBJECTS

The 100 young women reported in this study were all students at the University of Wyoming and were selected from a large number upon whom tests were made as being young women who were normal in every respect, as nearly as could be determined through records of their physical examinations and their health at the time the test was taken. Sixty-five per cent of the young women had lived in Wyoming all their lives, 30 per cent had lived in Wyoming more than 10 years, and the other 5 per cent came from other states, but no test was taken on a subject if she had lived at this altitude less than 6 months. All cases were discarded which showed any abnormality likely to affect the thyroid activity in any way or whose medical record contained any recent treatment for thyroid trouble. The ages ranged from 17 to 26 years, sixty-eight of the 100 falling within the 19 to 22 age groups. The weights fell within the ± 10 per cent of the Wood standards (cited by Rose, '29).

PROCEDURE

The usual procedure was followed. The young women were asked to come to the metabolism room at 7.30 or 8 o'clock without breakfast in time to rest from $\frac{1}{2}$ to $\frac{3}{4}$ hour before the test was taken, thus making the time after the last meal from 12 to 14 hours. No test was taken if the subject had not had

the usual amount of sleep the preceding night. Records of height, weight, and temperature were made, and the pulse rate was recorded a number of times during each test.

APPARATUS

A new Benedict Roth Kymographic apparatus was used, and usually three 6 minute tests were made, although occasionally if the first two tests were very regular and checked exactly, the third test was omitted. In three cases where the subject had difficulty in getting adjusted to the test as many as five or six tests were made. The lowest basal rate obtained was used, but enough tests were made to get two that corresponded almost exactly. Wilson soda lime was used and changed frequently to avoid error.

RESULTS AND DISCUSSION

The individual records, including deviations from the Du Bois (modified by Boothby and Sandiford) standards and the Harris-Benedict standards are shown in table 1.

The same information averaged according to age groups is given in table 2.

The comparison of Wyoming results with the Du Bois (modified by Boothby and Sandiford) standards is made with the original Du Bois standards in the Oklahoma and Florida studies, and the results would be less pronounced if the Wyoming tables were reduced to the Du Bois standards, since Boothby and Sandiford ('29) state that their standards are from 1 to 4 per cent lower for adults than the original Du Bois standards.

The calories per square meter of body surface per hour, using the height-weight formula, range from 33.59 to 37.87, with an average for the 100 cases of 35.61. This figure is 9.23 per cent above the Oklahoma average and 9.61 per cent above that of Florida.

The total calories per 24 hours vary from 1113 to 1748 with an average of 1368 for Wyoming women, which is 9.8 per cent above Oklahoma and 10.7 per cent above Florida. The

TABLE 1

Basal metabolism of Wyoming college women

NO.	AGE, YEARS	HEIGHT, CENTIMETERS	WEIGHT, KILOGRAMS	SURFACE AREA, SQUARE METERS	PULSE	TOTAL CALORIES PER 24 HOURS	CALORIES PER SQUARE METER PER HOUR	DEVIATION FROM STANDARD: Du Bois modified by Boothby and Sandiford	DEVIATION FROM STANDARD: Harris-Benedict
1	17	161	79.0	1.82	72	1475	33.76	— 9.7	— 6.7
2	17	173	60.0	1.70	66	1489	36.48	— 2.4	+ 1.3
3	17	153	49.0	1.43	66	1361	39.66	+ 4.2	+ 2.6
4	17	166	55.3	1.60	62	1177	30.63	—18.0	—15.2
5	17	158	57.7	1.57	65	1411	37.44	+ 0.1	— 0.6
6	17	159	62.5	1.62	60	1180	30.34	—18.8	—19.5
7	18	163	52.5	1.54	68	1321	35.72	— 4.2	— 3.9
8	18	162	56.3	1.58	65	1515	39.94	+ 7.0	+ 7.6
9	18	158	59.0	1.59	75	1374	36.01	— 3.4	— 3.7
10	18	166	68.5	1.75	60	1381	32.87	—11.6	— 9.9
11	18	166	62.5	1.68	54	1596	39.57	+ 6.1	+ 8.1
12	18	175	62.0	1.74	66	1553	37.18	0	+ 4.4
13	18	171	56.3	1.65	60	1208	30.49	—18.2	—15.3
14	19	155	53.7	1.50	80	1299	36.09	— 2.1	— 5.0
15	19	159	50.0	1.49	60	1207	33.74	— 9.3	— 9.8
16	19	150	46.0	1.39	60	1220	36.57	— 1.7	— 4.9
17	19	167	62.0	1.69	60	1485	36.61	— 1.5	+ 1.2
18	19	157	60.7	1.60	62	1399	36.42	— 2.1	— 2.6
19	19	172	57.0	1.66	66	1440	36.14	— 2.2	+ 0.8
20	19	160	49.5	1.48	58	1180	33.20	—10.7	—11.7
21	19	164	53.3	1.56	60	1270	33.90	— 8.8	— 7.9
22	19	174	66.8	1.79	60	1285	29.90	—19.5	—15.8
23	19	165	58.0	1.62	50	1479	38.04	+ 2.3	+ 3.7
24	19	164	63.0	1.66	60	1354	33.98	— 8.6	— 8.0
25	19	158	59.5	1.59	70	1550	40.61	+ 8.5	+ 8.8
26	19	151	44.3	1.35	52	1488	45.92	+23.4	+17.4
27	19	155	42.0	1.35	72	1241	38.28	+ 2.9	— 1.0
28	19	169	62.5	1.70	60	1588	38.82	+ 4.6	+ 7.7
29	19	151	36.3	1.25	66	1186	39.52	— 6.3	— 0.4
30	19	163	53.5	1.55	60	1274	34.24	— 7.7	— 7.6
31	19	157	48.0	1.45	63	1163	33.41	—10.1	—11.5
32	19	168	67.5	1.75	66	1442	34.32	— 7.7	— 5.3
33	19	170	66.5	1.75	72	1263	30.06	—19.0	—16.6
34	20	163	56.3	1.59	72	1348	35.32	— 4.2	— 3.8
35	20	167	74.0	1.81	60	1658	38.12	+ 3.4	+ 5.1
36	20	160	44.0	1.41	60	1279	37.80	0	0
37	20	160	47.0	1.45	66	1303	37.42	+ 1.4	— 0.4
38	20	156	54.5	1.52	72	1376	37.71	+ 2.2	± 0.3
39	20	174	57.5	1.67	60	1749	43.62	+18.2	+22.0
40	20	163	59.7	1.62	62	1428	36.73	— 0.4	— 0.3
41	20	163	53.0	1.55	68	1347	36.20	— 1.9	— 1.7
42	20	166	53.0	1.55	57	1216	32.48	—11.9	—11.6
43	20	160	54.3	1.54	72	1248	33.76	— 8.6	— 9.3
44	20	163	62.0	1.65	64	1395	35.22	— 4.5	— 4.2
45	20	160	45.0	1.42	68	1098	32.21	—12.7	—14.7
46	20	163	56.0	1.58	60	1225	32.30	—12.4	—12.4
47	20	167	67.5	1.75	66	1416	33.72	— 7.9	— 6.5
48	20	153	44.5	1.37	60	1341	40.77	+10.5	+ 5.8
49	20	166	80.0	1.86	60	1555	34.83	— 5.6	— 4.7
50	20	166	60.0	1.66	72	1260	[illegible]	6.0	4.8

TABLE 1—*continued*

NO.	AGE, YEARS	HEIGHT, CENTI-METERS	WEIGHT, KILO-GRAMS	SURFACE AREA, SQUARE METERS	PULSE	TOTAL CALORIES PER 24 HOURS	CALORIES PER SQUARE METER PER HOUR	DEVIATION FROM STANDARD: Du Bois modified by Boothby and Sandiford	DEVIATION FROM STANDARD: Harris-Benedict
51	20	160	48.0	1.46	72	1373	39.26	+ 6.4	+ 4.3
52	20	168	65.0	1.92	64	1504	36.43	— 2.8	0
53	20	169	65.0	1.72	60	1387	33.59	— 8.9	— 7.3
54	20	153	46.5	1.40	90	1353	29.10	+ 9.1	+ 4.9
55	20	161	53.5	1.55	58	1401	37.66	+ 2.0	+ 2.2
56	20	168	54.5	1.60	72	1280	33.33	— 9.7	— 8.5
57	20	160	61.0	1.62	48	1360	34.97	— 5.2	— 5.5
58	21	156	50.0	1.47	78	1324	37.53	+ 1.7	+ 0.1
59	21	168	61.0	1.67	66	1469	36.64	+ 0.7	+ 1.2
60	21	163	69.5	1.73	60	1379	33.20	—10.0	— 9.4
61	21	157	67.0	1.66	64	1436	36.03	— 2.3	— 3.5
62	21	152	50.5	1.44	60	1259	36.43	— 1.3	— 4.6
63	21	155	56.5	1.52	64	1525	41.80	+13.0	+10.2
64	21	165	55.5	1.60	66	1486	38.69	+ 5.3	+ 6.6
65	21	165	61.0	1.65	60	1376	34.74	— 5.8	— 4.7
66	21	176	62.5	1.75	66	1511	35.96	— 2.5	+ 2.0
67	21	169	62.8	1.70	66	1495	36.65	— 0.7	+ 1.8
68	21	164	53.0	1.56	60	1209	32.80	—12.5	—11.5
69	21	160	55.0	1.55	66	1173	31.52	—14.6	—14.2
70	21	166	58.5	1.63	64	1290	32.97	—10.6	— 9.4
71	22	154	49.0	1.43	72	1114	32.45	—12.0	—14.6
72	22	165	56.5	1.60	60	1600	41.67	+12.9	+14.5
73	22	166	59.0	1.63	56	1110	28.36	—23.1	—22.0
74	22	163	64.5	1.67	60	1441	35.94	— 2.5	— 2.1
75	22	161	52.3	1.52	66	1209	33.13	—10.2	—10.4
76	22	162	48.0	1.47	60	1394	39.52	+ 7.1	+ 6.3
77	22	165	54.5	1.58	60	1345	35.45	— 3.9	— 2.7
78	22	167	63.0	1.69	56	1421	35.06	— 5.0	— 2.9
79	22	165	52.0	1.55	66	1339	35.99	— 2.4	— 1.1
80	22	165	61.7	1.67	63	1382	34.49	— 6.5	4.4
81	22	152	56.0	1.51	70	1350	37.25	+ 1.0	— 1.3
82	23	162	57.0	1.59	60	1452	38.05	+ 3.1	+ 4.4
83	23	159	52.0	1.50	66	1579	43.86	+18.0	+17.9
84	23	159	64.5	1.64	66	1535	39.00	+ 5.6	+ 5.3
85	23	177	71.0	1.85	64	1575	32.47	— 3.9	+ 1.3
86	23	162	61.3	1.63	52	1259	32.17	—12.8	—12.1
87	23	159	48.0	1.46	60	1409	40.21	+ 9.0	+ 8.3
88	23	159	47.0	1.45	72	1203	34.57	— 6.3	— 6.4
89	23	168	68.0	1.75	50	1417	33.74	— 8.5	— 5.9
90	24	160	51.0	1.51	72	1256	34.65	— 6.1	— 5.4
91	24	162	56.3	1.57	82	1548	41.08	+11.3	+12.1
92	24	165	49.5	1.51	60	1346	37.15	+ 0.6	+ 1.8
93	24	158	57.0	1.56	60	1354	36.15	— 2.0	— 1.9
94	24	170	59.5	1.68	66	1495	37.08	+ 0.5	+ 4.8
95	24	165	67.0	1.72	66	1404	34.02	— 7.8	— 5.7
96	25	161	66.0	1.68	64	1289	31.97	—12.6	—12.1
97	25	166	56.0	1.61	58	1261	32.69	—10.8	— 8.6
98	25	167	52.5	1.57	72	1361	36.12	— 0.1	+ 0.8
99	26	159	57.0	1.55	60	1214	32.64	—10.8	— 4.2
100	26	168	58.0	1.65	64	1373	34.67	— 5.2	— 1.8

calories per 24 hours per kilogram of body weight vary from 18.66 to 30.37 with an average of 24.23, which is 7.7 per cent above Oklahoma and 6.2 per cent above Florida.

The average heat production per centimeter of height varied from 6.67 to 10 calories with an average of 8.4, which is 10 per cent above either Oklahoma or Florida.

TABLE 2

Basal metabolism of University of Wyoming women averages according to age groups

NUMBER OF CASES	AGE GROUP, YEARS	HEIGHT, CENTIMETERS	WEIGHT, KILOGRAMS	SURFACE AREA, SQUARE METERS	PULSE	TOTAL CALORIES PER 24 HOURS	CALORIES PER SQUARE METER PER HOUR	DEVIATION FROM STANDARD: Du Bois modified by Boothby and Sandiford	DEVIATION FROM STANDARD: Harris-Benedict
6	17	162	60.6	1.62	65.17	1349	34.72	— 7.4	— 6.3
7	18	166	59.6	1.65	64.00	1654	35.97	— 3.5	— 1.8
20	19	161	55.0	1.56	62.85	1340	35.99	— 3.8	— 3.4
24	20	163	56.7	1.59	65.12	1371	35.22	— 2.1	— 2.1
13	21	163	58.7	1.61	64.61	1379	35.76	— 3.0	— 2.7
11	22	162	56.0	1.57	62.63	1337	35.39	— 4.2	— 3.7
8	23	163	58.6	1.61	61.25	1429	36.76	+ 0.5	+ 1.6
6	24	163	56.7	1.59	67.66	1400	36.69	— 0.6	+ 1.0
3	25	165	58.2	1.62	64.67	1304	33.59	— 7.8	— 6.6
2	26	163	57.5	1.60	62.00	1294	33.65	— 8.0	— 3.0
Average		161	57.2	1.60	64.02	1368	35.61	— 3.2	— 2.5
Oklahoma						1245	32.6	—13.2[1]	—10.1
Florida		159	54.5		68.00	1236	33.4	—10.6[1]	— 9.9

[1] Du Bois.

Pulse rates varied from 48 to 90 with an average of 64 per minute, while respiration varied from 5 to 24 with an average of 13.8 per minute. No relation was found to exist between basal metabolism and either pulse or respiration.

SUMMARY

1. Basal metabolism rates were determined on a group of 100 normal college women students at the University of Wyoming, most of whom had lived in Wyoming more than 10 years. The average deviations calculated from prediction

standards are: Aub Du Bois, modified by Boothby and Sandiford, —3.18 per cent; Harris-Benedict, —2.54 per cent.

2. These metabolic rates are higher than rates found in Oklahoma and Florida under similar conditions, which would indicate that the high altitude, 7148 feet, raises the basal metabolism.

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A STUDY OF THE MAGNESIUM NEEDS OF PRESCHOOL CHILDREN

AMY L. DANIELS AND GLADYS J. EVERSON

WITH THE COOPERATION OF MARY F. DEARDORFF, ELIZABETH M. KNOTT, FLORENCE I. SCOULAR AND OLIVE E. WRIGHT

Iowa Child Welfare Research Station, State University of Iowa, Iowa City

TWO FIGURES

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The untoward symptoms following magnesium deprivation in rats observed by McCollum and Orent ('31), Kruse, Orent and McCollum ('32, '33), Kruse, Schmidt and McCollum ('33) and Greenberg and Tufts ('35) together with the findings of Duncan, Huffman and Robinson ('35) to the effect that tetany and death in calves receiving milk rations are related to the low magnesium content of the diet has led to an investigation of the magnesium needs of children. Hitherto, it has been assumed that diets consisting of a variety of foods contained sufficient magnesium, since no abnormal conditions which seemed related to a deficiency of this element have been noted. However, diets consisting of refined cereals and the generous amounts of milk advocated for children of the preschool period may contain less than the optimum amounts. In the process of refining, much of the magnesium is removed from the grain and milk is comparatively low in this constituent. Indeed, Hirschfelder ('34) has reported ten cases of low plasma magnesium associated with hyperirritability of the neuromuscular system, muscular twitchings and convulsions in patients receiving special soft diets which included considerable amounts of milk and other low magnesium containing foods.

The literature contains little information relative to the amount of magnesium needed by normal growing children. Chaney and Blunt ('25) have reported balance studies with two girls 10 and 11 years old, and Wang, Kaucher and Wing ('35), with twenty-three adolescent girls. In the former study, the magnesium retentions were related to the ingestion in a given child. There was, however, no consistent relation between the amounts ingested and retained in the two children studied. With the Wang, Kaucher and Wing studies, although the average ingestions were fairly comparable to those of Chaney and Blunt, the average retentions were somewhat less. These differences in retention may be concerned with the physical condition of the children studied, or with the fact that the diets differed in substances other than magnesium. Haag and Palmer ('28) have stressed that balanced conditions of calcium, magnesium and phosphorus are essential for normal growth.

EXPERIMENTAL PROCEDURE

In the investigation, thirty-three magnesium, calcium and phosphorus balance studies have been made with thirteen children (ten boys and three girls) between 4 and 7 years of age, receiving varying amounts of magnesium as well as calcium and phosphorus. Each balance study, with one exception, has consisted of a preperiod of 6 or 7 days to allow for adjustment to a given ingestion level, and a double metabolism period of 5 days each, thus minimizing unavoidable errors from inaccurate stool marking. The care of the children, preparation of the food and details of stool and urine collection were the same as reported in the previous study (Daniels et al., '35).

Each day's food consisted of milk, meat, eggs, potatoes, prunes, apple sauce, banana, carrots, breakfast cereal, bread, butter, and 1.5 gm. of iodized table salt, distributed among the three meals. Distilled water was used for drinking and cooking purposes. In all cases, the water used in cooking was evaporated and included in the food served. The 3.6 gm.

of a standard brand of cod liver oil plus 4 drops of viosterol twice daily, with a given amount of orange juice, and 120 gm. of canned tomato would seem to rule out any question of a vitamin D or vitamin C deficiency.

Variations in the level of magnesium were obtained by altering the amount of milk and cereals in the diets, and by using commercial whole grain bread and breakfast cereal in some of the diets and white bread and refined cereals in others. Precautions were taken to insure a constant level of magnesium in a given diet by using bread from a single loaf and cereal from the same package for an entire metabolism period for a given child. The other foods differed only in the amounts necessary to supply the protein and caloric needs of the children. The range of magnesium ingestions was not as wide as might be desired, due in part to the exigencies of other studies which were being carried on simultaneously and in part to the fact that in metabolism studies with children, diets must be planned which will be acceptable for a considerable period, since it is desirable that the same foods be prepared in the same way and taken in the same amounts during the period of study. Changes either in serving portion or preparation may result in confusion in interpretation. Furthermore, the diets used must be physiologically safe, since the children must be kept well during the study, and at the end be in as good condition as at the beginning, in so far as can be determined. Wide variations in the substances being tested, possible in animal nutrition studies, cannot be used for children for any considerable period.

The methods for the calcium and phosphorus determinations were the same as those of the former study (Daniels et al., '35). Magnesium determinations were made with filtrates from the calcium precipitates by the method described by Epperson ('28). To test the reliability of the method for the particular material under consideration a given quantity of magnesium (as anhydrous magnesium sulphate) was added from time to time to the aliquots used for analysis. Recov-

eries of from 96.1 to 100.1 per cent indicated that the method was satisfactory. Triplicate determinations were made in all cases. Results which seemed at variance were repeated.

RESULTS

Since the particular interest of the study is concerned with the magnesium needs of children, the magnesium balances have been listed in order of magnesium retentions with the corresponding calcium and phosphorus retentions and ingestions, to determine the possible influence of these on the magnesium metabolism.

In any nutrition studies with children, it is often difficult to determine to what extent the results have been modified by the nutritional condition of the child, or possibly by other factors in the diet. Studies with children differ from those with animals in that there are necessarily differences in potentialities of growth, due to different inheritances, long preperiods on a given diet are not possible, and wide variations in the substances being studied which might bring about untoward results cannot be tested. In order to determine, therefore, how much of a given substance is needed, it is desirable to study retentions with various levels of ingestion in a few children whose recent nutritional histories are known and have been controlled. Among the thirty-three balance studies reported, eight have been with one child (D.G.); with two exceptions, these were consecutive. Five balance studies with a second child (F.V.) also are listed. The dates indicate the consecutive periods for each child.

The magnesium ingestions of the children studied were between 11.3 mg. and 19.0 mg. per kilogram, whereas the magnesium retentions ranged between 0.4 mg. and 3.1 mg. per kilogram. Of these, 57.6 per cent were between 1.0 mg. and 2.0 mg. (average 1.38 mg. per kilogram), 30 per cent between 0.4 mg. and 1.0 mg. (average 0.6 mg.) and 12 per cent between 2.0 mg. and 3.1 mg. per kilogram (table 1). High retentions were not consistently associated with high ingestions, nor were low ingestions always followed by low

TABLE 1

The relation of magnesium retention to magnesium, calcium and phosphorus ingestion

CHILD	DATE	WEIGHT	MAGNESIUM Retention per kilogram	MAGNESIUM Ingestion per kilogram	MAGNESIUM Urine per kilogram	MAGNESIUM Feces per cent of ingestion	CALCIUM Ingestion per kilogram	CALCIUM Retention per kilogram	PHOSPHORUS Ingestion per kilogram	PHOSPHORUS Retention per kilogram	RATIO—Ca : Mg Ingestion	RATIO—Ca : Mg Retention
		kg.	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*		
C.H.	11/14	15.8	3.1	18.2	5.4	53	53.7	12.4	73.9	9.7	2.9	4.0
D.G.	11/14	19.3	2.8	15.0	3.8	56	43.3	10.5	62.5	7.7	2.9	3.8
F.V.	11/14	15.9	2.5	18.7	4.7	62	54.1	11.7	71.6	10.0	2.9	4.7
D.G.	1/16	19.8	2.0	12.2	3.7	53	41.1	9.9	53.1	5.8	3.4	5.0
F.V.	6/11	16.8	1.8	15.6	4.4	60	49.9	10.4	66.4	7.9	3.2	5.8
J.E.	10/ 1	15.8	1.8	17.0	3.9	66	86.2	9.9	83.1	7.7	5.1	5.5
G.B.	10/22	23.7	1.7	14.5	4.1	60	59.5	10.3	65.7	5.2	4.1	6.1
F.V.	2/ 5	16.8	1.7	15.0	4.2	61	80.0	7.8	80.5	6.9	5.3	4.6
B.B.	1/16	15.4	1.6	14.3	3.8	62	50.4	6.9	63.5	8.2	3.5	4.3
F.V.	1/16	16.4	1.6	13.7	3.7	61	50.0	7.9	59.3	8.4	3.6	4.9
J.F.	2/25	19.0	1.6	11.9	3.1	60	46.6	8.4	59.2	4.6	3.9	5.3
J.F.	2/ 5	18.7	1.5	13.7	2.5	71	73.4	8.0	75.7	3.5	5.4	5.3
M.Y.	10/ 1	13.2	1.4	19.0	3.3	75	100.0	15.4	96.4	10.7	5.3	11.0
J.E.	10/22	18.6	1.4	16.7	3.7	69	81.3	11.2	78.2	8.5	4.9	8.0
P.E.	5/22	16.1	1.3	14.0	4.1	61	84.4	5.2	86.0	1.2	6.0	4.0
F.V.	12/10	16.6	1.3	13.2	4.3	58	47.4	11.1	57.2	7.7	3.6	8.5
D.G.	6/11	20.4	1.2	13.3	3.8	62	43.4	10.0	59.2	5.5	3.3	8.3
D.G.	2/ 5	19.9	1.1	12.7	3.6	63	68.7	10.2	73.1	4.8	5.4	9.3
R.S.	10/ 1	21.3	1.1	14.2	3.9	65	67.0	8.6	68.3	4.8	4.7	7.8
J.H.	3/18	15.7	1.1	14.4	3.8	66	51.2	6.0	67.5	2.9	3.6	5.5
P.E.	4/30	16.2	1.1	14.4	4.8	59	89.2	11.1	94.1	5.7	6.2	10.1
D.G.	2/25	19.9	1.0	11.3	3.4	61	44.7	7.4	55.7	3.3	4.0	7.4
R.S.	10/22	22.2	1.0	13.9	3.8	65	63.0	8.6	64.4	2.7	4.5	8.6
D.G.	5/20	20.0	0.9	11.8	3.4	64	70.2	6.9	77.3	3.8	5.9	7.7
J.E.	4/ 8	17.0	0.9	13.0	3.7	65	81.0	17.5	75.4	8.3	6.2	19.4
M.Y.	4/30	14.0	0.8	14.9	3.7	70	97.7	11.1	84.3	15.7	6.6	13.9
J.E.	3/18	16.7	0.8	13.6	3.1	71	48.7	9.9	66.5	4.4	3.6	12.4
D.G.	4/30	19.8	0.6	11.9	3.4	66	72.0	10.3	74.9	7.6	6.1	17.1
D.G.	3/18	19.9	0.6	11.6	3.2	67	41.5	6.5	56.3	1.4	3.6	10.8
D.B.	6/11	20.4	0.5	13.2	3.8	67	43.3	9.6	61.9	4.5	3.3	19.2
J.N.	12/10	14.0	0.5	14.6	3.9	70	56.0	5.8	65.5	6.4	3.8	11.6
J.H.	4/ 8	16.5	0.5	13.4	4.7	61	84.4	11.2	79.2	6.2	6.3	22.4
M.Y.	5/20	14.5	0.4	13.6	3.6	71	91.6	12.1	81.1	4.6	6.7	30.3

retentions. A consideration of D.G.'s retentions during eight metabolism periods and of F.V.'s during five (table 2) suggests that the high retentions in some of the children studied were due to previous depletion since subsequent retentions with ingestions which would seem to be adequate were lower. On the other hand, the lowest ingestions were coexistent with low retentions indicating possibly that these ingestions were

TABLE 2

The relation of magnesium ingestion to magnesium retention

CHILD	DATE	WEIGHT	MAGNESIUM Intake per kilogram	MAGNESIUM Urine per kilogram	MAGNESIUM Feces per kilogram	MAGNESIUM Retention per kilogram
		kg.	*mg.*	*mg.*	*mg.*	*mg.*
D.G.	11/14	19.3	15.0	3.8	8.4	2.8
	1/16	19.8	12.2	3.7	6.6	2.0
	2/ 5	19.9	12.7	3.6	8.0	1.1
	2/25	19.9	11.3	3.4	6.9	1.0
	3/18	19.9	11.6	3.2	7.8	0.6
	4/30	19.8	11.9	3.4	7.9	0.6
	5/20	20.0	11.8	3.4	7.5	0.9
	6/11	20.4	13.3	3.8	8.3	1.2
F.V.	11/14	15.9	18.7	4.7	11.6	2.5
	12/10	16.6	13.2	4.3	7.7	1.3
	1/16	16.4	13.7	3.7	8.4	1.6
	2/ 5	16.8	15.0	4.2	9.1	1.7
	6/11	16.8	15.6	4.4	9.4	1.8

below the optimum needs. During four periods, D.G. (table 2) retained between 0.6 mg. and 1.0 mg. per kilogram. Following a considerable period of low ingestions, the effect of a higher intake was tested with the result that more was retained. F.V.'s retentions also (table 2) correlate very closely with ingestions, although at no time did he receive the low ingestions of D.G., nor were his retentions so low. Among the group with low retentions, 0.4 mg. to 0.9 mg. per kilogram, there were seven children who were receiving fairly

high ingestions. Were these lower retentions an expression of lesser need, or was the magnesium less available due to more rapid passage through the tract or some unappreciated digestive disturbance? The percentage of magnesium in the feces, based on intake, of some of this group was somewhat higher than among the children with higher retentions. Since magnesium is eliminated by way of the tract as well as through the kidney, at present there is no way of determining what proportion of the fecal magnesium is unabsorbed and what proportion is excreted magnesium.

That some of the low magnesium retentions were the result of a too low magnesium ingestion is suggested by the urinary magnesiums. D.G. excreted in the urine 3.2 mg. and 3.4 mg. per kilogram with ingestions of 11.9 mg. per kilogram or less (table 2), whereas with the higher ingestions previously tested, the urinary magnesium was consistently higher; F.V., receiving between 13.2 mg. and 18.7 mg. per kilogram and retaining between 1.3 mg. and 2.5 mg., excreted in the urine from 3.7 mg. to 4.7 mg. per kilogram. The relation of low urinary magnesium to less than optimum ingestions is also suggested by the results of Wang, Kaucher and Wing, and Chaney and Blunt. With an average ingestion of 8 mg. per kilogram (considerably less than was included in the diets of the preschool children studied) Wang, Kaucher and Wing report an average urinary output of 3.4 mg. per kilogram with an average retention of 0.4 mg. per kilogram, while Chaney and Blunt found from 2.5 mg. to 3.3 mg. per kilogram in children receiving 7.3 mg. and 10.1 mg., respectively, and retaining 1.7 mg. to 3.4 mg. per kilogram, and from 3.6 mg. to 4.0 mg. with higher ingestion levels, 10.1 mg. to 13.6 mg. per kilogram, respectively. Retentions following the higher ingestions also were higher, 2.8 mg. to 5.0 mg. per kilogram.

Assuming that high urinary magnesiums (over 3.4 mg. per kilogram) are indicative of a sufficient ingestion, then the children of the study who were retaining between 0.4 mg. and 0.9 mg. of magnesium per kilogram and were excreting over 3.4 mg. of urinary magnesium per kilogram (60 per cent of

the low retention group) were receiving sufficient for their physiologic needs. If this interpretation is correct, it must be concluded that a large proportion of the children studied had been receiving previously less than their optimum needs since 70 per cent retained 10 mg. per kilogram or more. There may be, however, other explanations not yet apparent for these low retentions.

Considering ingestions in relation to retentions and urinary magnesium, and drawing conclusions from the data at hand, it would seem that 13 mg. per kilogram of body weight is the least amount of magnesium that should be included in the diets of children of the ages studied. Further studies with other types of diets are needed to establish the validity of this conclusion. It is interesting to note, however, that the high average of magnesium in the 3000 calory diet reported by Sherman ('28) when considered in relation to the caloric intake of these children, is very comparable to this figure.

THE RELATION OF MAGNESIUM TO CALCIUM AND PHOSPHORUS METABOLISM

The interrelation of the calcium and magnesium salts postulated by the early workers seems not to have been borne out by recent research, excepting in those studies with animals where comparatively large amounts of magnesium salts either were fed or injected, and even in these the results may have been wrongly interpreted, since the introduction of an excessive amount of magnesium into the ration "resulted in such reduced palatability and lowered food intake, accompanied by such severe digestive disturbance that any specific effect of magnesium or calcium relation in the body was masked" (Elmslie and Steenbock, '29); and the untoward effects noted in conditions where the phosphorus was low or where the magnesium salts were injected may have been due to basicity, rather than to the magnesium salts per se, for with seemingly normal diets the calcium, phosphorus, and magnesium may be varied within rather wide ranges without producing visible abnormalities (Medes, '26). Furthermore,

in calves suffering from tetany, Duncan, Huffman and Robinson ('35) observed that although the serum magnesium was considerably below normal, the calcium and phosphorus were well within the normal range. Similarly, Denis and Talbot ('21) found in children suffering from various types of diseases low plasma magnesium with normal calcium values. Convulsions noted in certain cases were associated with both low magnesium and low calcium. The addition of vitamin D was without influence on the magnesium blood values, suggesting that there is little causal relationship between these elements in the blood (Duncan, Huffman and Robinson).

In studies with the human organism it was noted that the addition of magnesium lactate to the diets of two adult males was followed by a very definite increase in calcium retention in one case and a decrease in another, while magnesium retentions were significantly increased in both (Carswell and Winter, '31). Magnesium citrate also caused a marked increase in magnesium retentions, and a definite decrease in calcium retentions in the four subjects studied by Bogert and McKittrick ('22). Coons and Coons ('35), on the other hand, were unable to find any influence of variations in magnesium ingestion on the calcium and phosphorus metabolism during successive studies in a gravid human subject and conclude that "magnesium metabolism is not antagonistic to that of calcium where the intake amounts to no more than is found in ordinary dietaries."

The relation of magnesium metabolism to calcium and phosphorus metabolism in the children of the present investigation has been studied: 1) by comparing the calcium: magnesium ingestion ratios with the calcium: magnesium retention ratios, and 2) by comparing the magnesium and phosphorus retentions with the calcium retentions arranged in order of calcium retentions.

A consideration of these calcium: magnesium ingestion ratios suggests that they were not the influencing factors, either in the magnesium or calcium retentions. The ingestion ratios were between 2.9 and 6.7 whereas the retention

ratios ranged from 3.8 to 30.3, 39 per cent being between 3.8 and 5.8 inclusive, 39 per cent between 5.8 and 11.6, and 21 per cent over 12. Low retention ratios were not co-existent with low ingestion ratios, nor did high ingestion ratios result in high retention ratios. Indeed, in the children studied, there seemed to be little if any interdependence of calcium and magnesium metabolism (fig. 1). Apparently some of the children studied needed more calcium, whereas some needed more magnesium. Nor did the phosphorus metabolism appear to influence magnesium retentions; at least, there seemed to be no general trend indicating a direct relationship, whereas the relation between calcium and phosphorus retentions was very apparent (fig. 1). The relationship between calcium and phosphorus and the lack of relation of magnesium with either is further emphasized by the two children who were studied during consecutive periods (fig. 2). When the children first came to the ward, they seemingly had been receiving previously less than the optimum amount of all three metabolites for subsequently their retentions were lower. During four periods, D.G. received less than the optimum amount of magnesium since the urinary output was low and subsequently increased retentions were obtained at a higher level. Phosphorus and calcium retentions also decreased during these periods, but these obviously were not caused by low ingestions since during two periods of low retentions the ingestions were high. Only one period (June 11th) showed higher retentions of all three metabolites with higher magnesium ingestions, which suggests the possibility that under certain conditions the magnesium of the diet may be an influencing factor in calcium and phosphorus metabolism. Previously, however, equally high calcium retentions as well as high phosphorus retentions had been obtained with lower magnesium ingestions. There were fewer consecutive studies with F.V., and because of this, interpretation is difficult. The higher retentions of both phosphorus and magnesium would seem to indicate that his home diet was less satisfactory than D.G.'s. On the other hand, the magnesium ingestion may

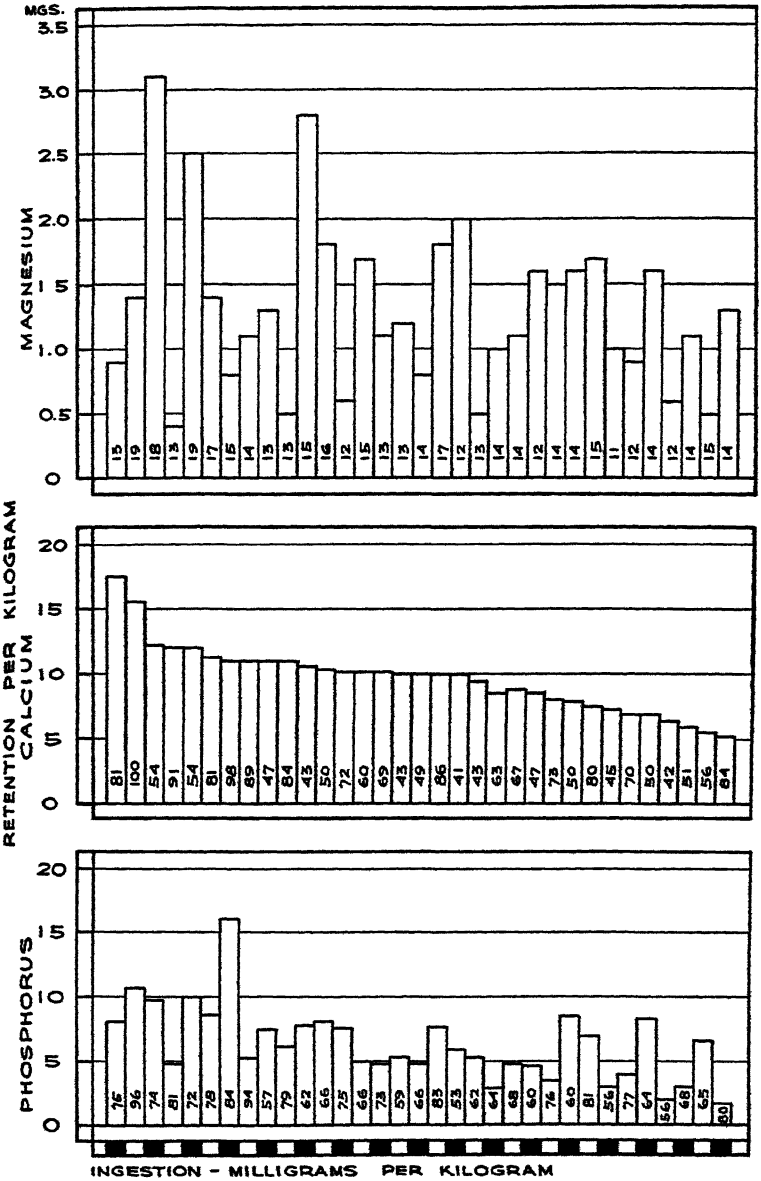

Fig. 1 Corresponding magnesium, calcium, and phosphorus retentions arranged in order of calcium retentions show no consistent interrelation.

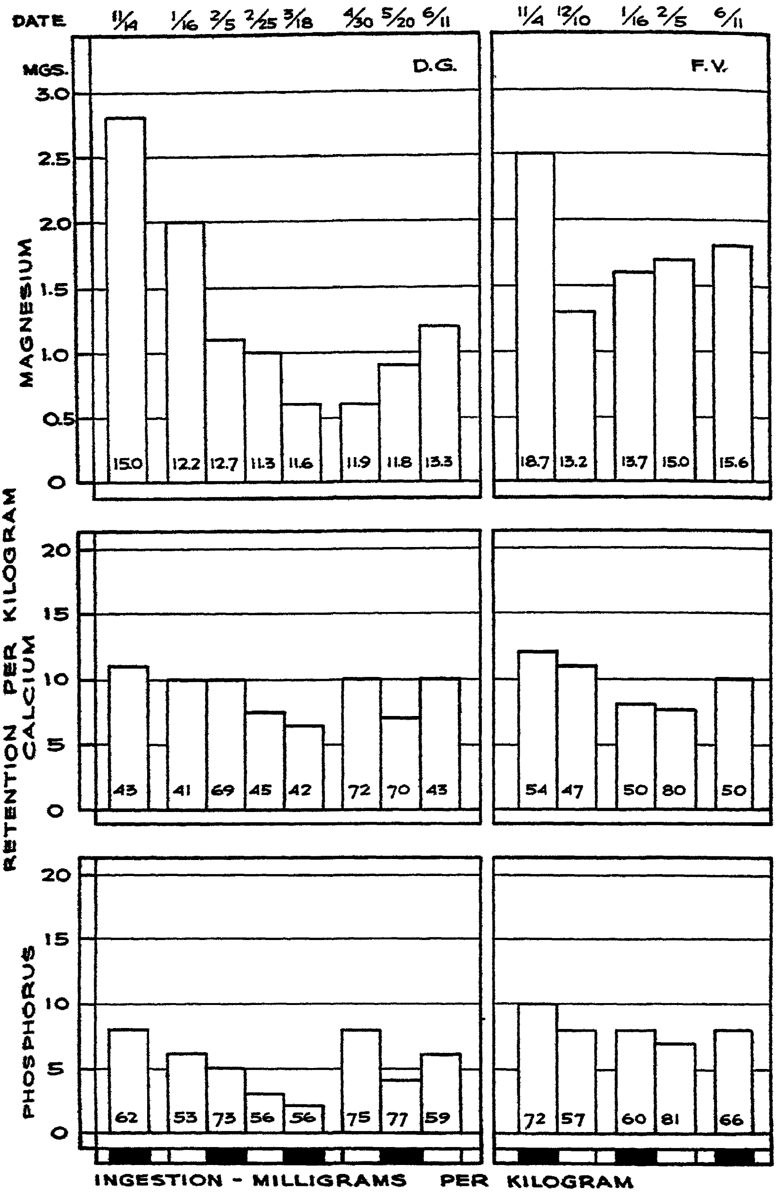

Fig. 2 Magnesium, calcium and phosphorus retentions during consecutive metabolism periods of D.G. and F.V.

have been more nearly optimum during all periods. Again calcium and phosphorus retentions run parallel, whereas magnesium retentions parallel the calcium and phosphorus only in the last period, June 11th, after 4 months at home where the diet may have been low in all three constituents.

From the data available, there is little evidence that either calcium or phosphorus was a factor influencing the magnesium retentions in the children studied.

SUMMARY

In an attempt to determine the amount of magnesium needed by growing children, thirty-three balance studies have been made with children between 4 and 7 years of age receiving various levels of magnesium. Calcium and phosphorus balance studies were made simultaneously in order to determine to what extent these may have influenced the magnesium retentions. In the study, the influence of insufficient vitamin D and vitamin C was seemingly ruled out since the children received a constant and what was believed to be an adequate amount of vitamin D obtained from cod liver oil and viosterol, and vitamin C from canned tomato and orange juice given twice daily.

Magnesium retentions, which varied from 0.4 mg. to 3.1 mg. per kilogram, were considered in relation to ingestion, urinary magnesium, and the influence of the calcium:magnesium ingestion ratios. There was no evidence that the magnesium retentions were influenced by the calcium ingestions, nor was there seemingly any relation between the magnesium retentions and calcium retentions. Calcium:magnesium ingestion ratios ranged between 2.8 and 6.7, whereas calcium:magnesium retention ratios were between 3.8 and 30.3. The magnesium of the urine, which tended to parallel the magnesium ingestion suggested that this might be used as a means of determining the sufficiency of the diet in this respect. Low urinary magnesiums when co-existent with low retentions were indicative of too low ingestions. High urinary magnesiums with low retentions were interpreted as indicat-

ing that enough had been fed and that previously the individual had been receiving a diet containing a sufficient amount, whereas high urinary magnesiums with high retentions following high ingestions indicated that the individual had been receiving previously less than the optimum amount. Seventy-five per cent of the children studied were in this group.

It was concluded tentatively that diets of children of the ages studied should contain not less than 13 mg. per kilogram of body weight. Further studies with different types of diets are needed to confirm these findings.

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STUDIES ON THE RELATION OF DIET TO GOITER

III. FURTHER OBSERVATIONS ON A GOITROGENIC DIET

ROE E. REMINGTON AND HAROLD LEVINE[1]
Medical College of the State of South Carolina, Charleston

ONE FIGURE

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In 1933 (Levine, Remington and von Kolnitz, '33 a) we published results showing that it was possible to produce enlarged and hyperplastic thyroids in young rats fed for 5 weeks on a diet, which, while not in all respects perfectly balanced, was extremely low in iodine. This diet was identical with that employed by Steenbock to produce rickets in the rat, except that 0.2 per cent of irradiated yeast was added to prevent development of rickets. The choice of a rachitogenic diet was not deliberate on our part. We spent considerable time making iodine analyses of various foods, in an attempt to devise an otherwise adequate diet that would be sufficiently low in iodine for our purpose. It was only after obtaining discouraging results in other directions that we decided to follow up the observation of Krauss that rats on the Steenbock diet 2965 developed enlarged thyroids. Our use of this diet may have been unfortunate, in that it has led several writers to assert that the abnormal Ca : P ratio of the diet played a part in the experimental results, a question which will be discussed later in this paper.

We also showed that the addition to the diet of 3 γ per day, or less, of iodine as potassium iodide, effectively prevented the development of goiter. On this diet, the average fresh thyroid weight, per 100 gm. body weight, of 193 rats was

[1] Now with Premier Pabst Corporation, Milwaukee, Wisc.

53 mg. against 12 mg. for rats receiving added iodine, the dry matter content of the gland 19 per cent against 28 per cent, and the iodine content, dry basis, 0.008 per cent against 0.267 per cent. The diet contained 14 to 17 parts per billion of iodine, furnishing about 0.15 γ per rat per day.

Experiments to determine the iodine requirement of the rat (Levine, Remington and von Kolnitz, '33 b), showed that as the iodine intake was increased from 0.14 to 0.5 γ per day, the size of the gland decreased very rapidly, and when the iodine intake was further increased, the size decreased more slowly, reaching normal value at around 2 γ iodine per day. There was a definite positive correlation also between iodine intake and iodine content of the gland, and an inverse correlation between iodine intake and dry matter content. In the experiments reported, and a long series of others not as yet published, these correlations had always been found to exist so that it seems evident that under our experimental procedure, the iodine intake of the animals is indicated rather definitely by either the fresh weight, the dry matter content, or the iodine content of the gland.

The characteristic appearance of these glands on gross and histological examination, as described in the previous paper, is invariably seen in glands containing 0.08 per cent or less of iodine, dry basis, frequently but not universally in those containing up to about 0.12 per cent, but never in glands whose iodine content was as much as 0.2 per cent. Judged by these criteria, i.e., physical appearance and iodine content, it has frequently happened in our experiments that we could class glands as hyperplastic even when the enlargement was not so great as to appear, of itself, significant.

Other recent experiments on the feeding of low iodine diets have been reported by Jackson and P'An ('32), Thompson ('32, '33), Hibbard ('33), Coplan and Sampson ('35), Hellwig ('31, '35), and Sugiura and Benedict ('35). Thompson fed the Steenbock rachitogenic diet, and the same diet modified by the substitution of wheat germ for a part of the wheat gluten and by varying the calcium content. She observed by

histological methods that while iodine deficiency always resulted in hyperplasia, increasing the calcium content of the diet increased the severity of the condition. Adding iodine to the diets resulted in normal glands. Thompson's basal diet contained 30 parts per billion of iodine, and consequently probably yielded to her rats (food intakes not reported) 0.28 to 0.30 γ of iodine per day; an amount which, if fed for 35 days, according to our previously published results, should have yielded glands of double the normal size, and an iodine content of 0.0165 per cent, dry basis.

Thompson also found that the thyroid of rats fed a low iodine diet for 3 months or more underwent atrophy. In the preliminary work which led up to our selection of 35 days as the standard experimental period, we observed that rats fed our diet GP for longer periods did not show any greater degree of enlargement, and in fact, if the period were too long, the enlargement per 100 gm. body weight was apt to be less. Coplan and Sampson also observed secondary atrophy of the gland following hyperplasia on a low iodine diet. Their diet, however, used casein as a source of protein, and Osborne-Mendel salt mixture, hence was not rachitogenic.

Sugiura and Benedict, studying the effect of our diet GP on malignant growth, also observed that it was goitrogenic. However, they reported that all animals lost weight on the diet continuously until death, which occurred, on the average, in 46 days. This is so at variance with the experience of Thompson ('32), and of ourselves, that we are of the opinion that either the wheat gluten or the corn meal may have contained a toxic factor. Their animals only ate 2 gm. of food per day, whereas ours consumed 10 gm. on the average, and made weekly gains of 10 to 12 gm.

Jackson and P'An, and Hellwig, were unable to produce thyroid enlargement by low iodine diets. Analysis of the diets is not reported, but the cod liver oil concentrate employed by Jackson and P'An undoubtedly contained iodine, and other ingredients may have. Their experimental period was 7 months, and that of Hellwig 4 months, so that in both

cases they were working within the range in which Thompson and Coplan and Sampson found that secondary atrophy takes place.

A. RELATION AMONG WEIGHT, DRY MATTER AND IODINE CONTENT OF GLANDS

In support of the previously mentioned correlation among size of gland, iodine content of gland, and percentage of dry matter, we have collected and tabulated the results of all our experiments on which we have complete data, over a period of 4 years. The data consist of 125 experimental groups, totalling 1021 animals. Of this number, sixty-one groups were fed our standard goitrogenic ration or modifications of it which did not involve the addition of iodine. Nineteen experimental groups received the goitrogenic ration supplemented by varying amounts of iodine as potassium iodide. Twenty-four groups received the goitrogenic diet supplemented by various samples of dried foods which furnished known amounts of iodine. Ten groups were fed Russell's modification of the Sherman breeding diet, some of them with added iodine, and eleven groups received various purified diets which it was hoped might prove to be goitrogenic.

In figure 1 we have plotted fresh thyroid weight per 100 gm. body weight against percentage of iodine (dry basis) and against percentage of dry matter in the gland. In our previous work (Levine, Remington and von Kolnitz, '33 b) on iodine requirement in the rat, we concluded that between 1 and 2 γ per day would suffice to prevent thyroid enlargement, these daily intakes corresponding, respectively, to an iodine content in the dried gland of 0.115 and 0.177 per cent. The iodine curve in figure 1 is particularly interesting in view of this observation and also in view of the widely quoted statement of Marine that when the human thyroid contains less than 0.2 per cent of iodine (dry basis) goiter is present. Now while histological examination has revealed hyperplasia in rat thyroids containing 0.100 to 0.200 per cent of iodine, it appears from the graph that a marked increase in size does not occur until the iodine

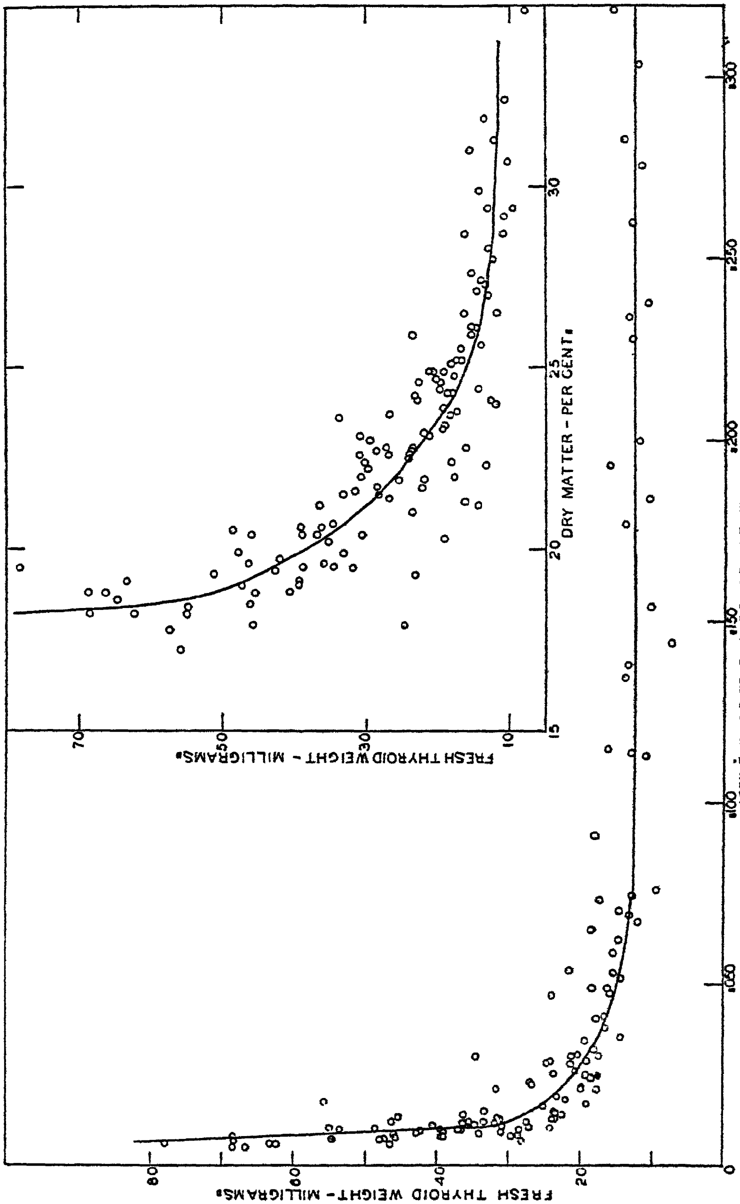

Fig. 1 Showing relationship of fresh weight with iodine content and dry matter content of thyroid glands of rats on iodine adequate and iodine deficient rations. Thyroid weight in milligrams per 100 gm. body weight.

content becomes as low as 0.075 per cent, or approximately one-third the value which we and others have considered as indicating adequate function.

That this correlation as to size of gland, iodine content, and dry matter content, exists also as to human glands has been shown by von Kolnitz and Remington ('33).

If it is desired to produce iodine deficient goiter in experimental animals for the purpose of studying the availability of certain natural food sources of iodine, or the effect of superimposed factors such as calcium, insanitary surroundings, light, and temperature, all of which have been thought to be contributing factors, then it appears from the data of figure 1 that it would be desirable to keep the iodine intake so low that the percentage in the dried gland will be below 0.05, or still better, below 0.03, so as to get within a critical range of hyperplasia. This probably corresponds to an iodine intake of 0.4 to 0.3 γ per day per rat, and is not easy to attain. A diet for the purpose would contain less than 35 parts per billion of iodine.

Although some lots of our GP diet showed as little as 15 parts per billion, the iodine content of corn is known to be variable, as is presumably that of wheat gluten. The analysis of each individual batch of diet by a procedure which requires the combustion of a kilogram or more in a closed system (which is both laborious and expensive and none too accurate for these minute traces) presents a tremendous obstacle. To attempt to repurify ingredients so as to remove such minute traces of iodine appears well-nigh impossible.

Furthermore, factors of the environment other than the diet itself may provide iodine. Thompson ('33) noted that if animals receiving an adequate iodine supply are kept near those on the deficient ration, the results on the latter were decreased or irregular, and urged the importance of strict segregation. If animals are kept in a building where other laboratory work is done, there is a possibility of their deriving iodine from air or dust. That this latter suggestion is not without reason is shown by table 1, in which we have

recorded the results of successive trials on our GP diet over a period of 4 years. In the winter of 1931–1932 we installed a chemical laboratory on the second floor of our building, which connects by an open stair-well with the animal rooms on the third floor. Subsequent to that date we have not been able to obtain as large glands as previously. Neither have they been as low in iodine or dry matter. This leads us to believe that our rats during this latter period have been getting somewhat more iodine from an unknown source than earlier.

TABLE 1

Results of successive feeding of diet GP to rats

DATE	NUMBER OF RATS	AVERAGE GAIN IN WEIGHT IN 35 DAYS	FRESH THYROID WEIGHT PER 100 GM. BODY WEIGHT	DRY MATTER IN THYROID	IODINE IN THYROID
		gm.	*mg.*	*per cent*	*per cent*
1930–1931	193	47	53.2 ± 0.9	19.0	0.0083
Feb. 1932	8	63	39.4 ± 1.7	19.1	0.0084
June 1932	9	59	23.9 ± 0.9	22.7	0.0152
Aug. 1932	9	42	24.3 ± 0.8	24.1	0.0286
Nov. 1932	8	60	24.2 ± 1.3	22.6	0.0103
Apr. 1933	9	39	30.6 ± 1.7	20.4	0.0109
Nov. 1934	15	65	29.1 ± 1.3	21.5	0.0220
KI added to furnish 3.72γ iodine per rat per day					
1930–1931	24	48	12.6 ± 0.19	28.0	0.2671
June 1932	9	55	12.1 ± 0.12	31.3	0.2000
Nov. 1932	5	42	14.3 ± 0.52	29.2	0.2828
Apr. 1933	9	33	13.2 ± 0.27	29.4	0.2600

Of course during this period we used numerous fresh lots of whole yellow corn, obtaining it from different known sources, and made repeated analyses of the mixed diets, without discovering wide variations in iodine content. In table 2 are shown iodine values for several lots of this diet, and from which it appears that the variations shown in table 1 cannot be due to changes in the iodine content of the diet itself. Neither have we been able to detect any measurable amount of iodine in our distilled water. Since 1931 we have received, dried, and ground numerous samples of sea food, however, and although we took such precautions as were possible, it

is not beyond the realm of possibility that dust from the grinding of these iodine-rich materials may have found its way to our animal and diet rooms. We agree with Thompson that if iodine intake is to be kept low enough to assure a significant and consistent degree of thyroid enlargement, the animals must be segregated from those receiving iodine supplements. We also feel that they should be segregated from chemical laboratories in which iodine in various forms may be used, and from the dust arising from the drying or grinding of iodine-rich substances.

TABLE 2

Analysis of successive lots of goitrogenic diet

DATE	IODINE	REMARKS
	parts per billion	
Jan. 1931	17	Usual rachitic diet
April 1931	20	Usual rachitic diet
	19	Rachitic diet from Fleischmann laboratories
Nov. 1931	14	With irradiated yeast 0.2 per cent
March 1932	15	With irradiated yeast 0.2 per cent
Sept. 1932	13	With irradiated yeast 0.2 per cent
Sept. 1932	20	Using commercial water-ground corn meal
Nov. 1932	14	Using corn from Ohio Experiment Station
Nov. 1932	14	Using corn from Minnesota Experiment Station
March 1933	13	
March 1933	11	Without irradiated yeast
May 1933	14	New supply of gluten

Although it may not be possible to know and control rigidly the iodine intake of rats on low iodine diets, experimental work can still be done with regard to the effect of various superimposed factors on low iodine goiter, provided, 1) the iodine deficiency is great enough to come within the critical range; 2) all animals are fed from the same batch of basal diet; 3) the handling of the animals is otherwise the same; and 4) the results as to size of gland, iodine content of gland, and dry matter content of gland, of the rats on basal diet, are in accord with the curves shown in figure 1.

With these precautions in mind, we have not found it difficult to produce glands weighing from 20 to 35 mg. per 100 gm.

body weight by feeding our GP diet for 5 weeks to 60 gm. rats: these glands will contain from 0.03 to 0.01 per cent of iodine, dry basis. If, then, one wishes to use this diet as a basis for experiments to determine the effect of varying different factors of the diet or environment, or the anti-goitrogenic value of foods, it is imperative that both positive and negative controls be run on the same batch of diet at the same time, and without variations in the environment of any group of animals.

B. EFFECT OF INCREASED IODINE IN BREEDING RATION

According to our technic, young rats of 60 gm. average weight (about 28 days old) are placed for 5 weeks on the experimental diet, at the end of which time they are killed by chloroform and the glands examined. We have previously reported (Levine, Remington and von Kolnitz, '33 a) that thirty-nine rats maintained for 9 weeks from birth on our colony ration, consisting of ground whole wheat, dried milk, dried meat scrap, and sodium chloride, yielded glands with an average fresh weight of 12.9 mg., dry matter content 23.3 per cent, and iodine content (dry basis) 0.0634 per cent. The iodine content of the colony ration varied from 47 to 72 parts per billion, but the average daily intake per rat per day during the 5 weeks experimental period was 0.74 γ. The glands were not enlarged, but the dry matter and iodine content were below normal, and some microscopic evidences of early hyperplasia appeared. From this we concluded that our colony ration was slightly inadequate as to iodine, and decided to determine what effect, if any, would result from increasing the iodine supply during the early life of the rats, and before they were placed on the goitrogenic diet.

The experimental procedure was as follows: One group of young rats was raised on the regular colony ration, and transferred to the goitrogenic ration at an average weight of 60 gm. A second group was raised on the colony ration, but potassium iodide was added to the drinking water so as to yield 1 γ of iodine per cubic centimeter, and when these rats

had reached 60 gm. in weight they also were placed on the goitrogenic ration. A third group of rats, raised on the colony ration, were placed on the goitrogenic ration supplemented by a fully adequate amount of iodine.

The results, shown in table 3, indicate that enhancing the iodine intake during the first 4 weeks of life does not enable the rats to store enough iodine to prevent the development of goiter when later deprived of this element.

TABLE 3

Effect of iodine content of breeding ration

DATE	NUMBER OF RATS	BREEDING RATION	EXPERIMENTAL RATION	DAYS ON EXPERIMENT	INITIAL AND FINAL WEIGHT	FRESH THYROID WEIGHT	DRY MATTER IN THYROID	IODINE IN THYROID (DRY BASIS)
					gm.	*milligrams per 100 gm. body weight*	*per cent*	*per cent*
October 1932	15	Stock	GP	35	64–135	25.5±0.82	22.6	0.0105
	15	Stock+KI	GP	35	59–115	23.3±0.36	24.2	0.0146
	5	Stock	GP+KI	35	60–111	14.3±0.52	29.2	0.2828

C. EFFECT OF CALCIUM

Several investigators have pointed to the high calcium content and abnormal Ca:P ratio (over 4:1) of our GP diet as being a factor, if not the predominating factor, of its goitrogenic power. Thompson ('33) reported on a diet quite similar to our own, that while thyroid hypertrophy occurred on the diet, more goiters developed, and they were larger, when the diet contained 3 per cent of added calcium carbonate than when it contained none. Hellwig ('35) added 2 per cent calcium chloride to a diet of corn meal and rolled oats (which should be very low in iodine) and found that goiter developed whether or not iodine was added to the diet, the goiters developing on a high iodine diet, however, being of the colloid rather than the parenchymatous type. An examination of the purest grade of calcium chloride we could purchase revealed 3300 parts per billion of iodine, hence the addition of 2 per cent of this salt would add approximately 0.66 γ per day to the iodine supply of the rat, which, together with the iodine

already present in the diet, might conceivably provide 0.8 to 1.0 γ per day, an amount which might be expected to remove iodine deficiency as a major factor in interpreting the experimental results. Our experience with varying the calcium content and the Ca : P ratio of our diet, is so at variance with that of these workers, that we feel that considerable more work must be done before the discrepancies can be explained, especially in view of the finding of Hibbard ('33) that sodium chloride, as well as calcium chloride, can produce the hyperplasia.

TABLE 4

Effect of varying calcium intake and Ca: P ratio

DATE	NUMBER OF RATS	DURATION OF EXPERIMENT	DIET	$CaCO_3$	Ca : P RATIO	INITIAL AND FINAL BODY WEIGHTS	FRESH THYROID WEIGHT	DRY MATTER IN THYROID	IODINE IN THYROID (DRY BASIS)	
		days		*per cent*		*gm.*	*milligrams per 100 gm. body weight*	*per cent*	*per cent*	
February 1932	8	35	GP	1	1.25	61–132	45.6 ± 2.6	18.9	0.0078	
	8	35	GP	3	4.1	65–128	39.4 ± 1.7	19.1	0.0084	
	8	35	GP	4.5	6.2	64–111	34.7 ± 2.2	19.5	0.0113	
April 1933	9	35	Rachitic	3	4.1	65– 92	36.6 ± 1.3	21.2	0.0102	Rickets
	9	35	Rachitic	1	1.25	62–104	45.9 ± 3.9	20.4	0.0087	
	9	56	Rachitic	3	4.1	67– 97	31.0 ± 1.3	22.6	0.0125	Rickets
	9	56	Rachitic	1	1.25	64–122	33.9 ± 2.7	23.6	0.0088	

In table 4 are shown the results of two experiments in varying the calcium intake and Ca : P ratio of animals on the goitrogenic ration. Certainly the evidence does not show larger glands or lower iodine when the calcium intake is increased, but rather the reverse. In the first experiment the difference in size of gland between rats fed 1 per cent of $CaCO_3$ and those fed 4.5 per cent appears to be significant (significance ratio 3 : 1) as does the iodine content. It may be that the $CaCO_3$ itself carried a trace of iodine. No histology was done on these glands, hence our findings cannot be considered as in complete opposition to those of Thompson until both chemical and histological examinations are made on the same group of animals.

Since completing the above experiments, we have gone back and reviewed some preliminary work done in April, 1931, and before we had adopted irradiated yeast as a source of vitamin D. In these experiments we fed the usual rickets-producing diet, but added various salts, phosphates, or phosphoric acid, or calcium chloride, in order to change both the Ca: P ratio

TABLE 5

Effect of varying Ca: P ratio and acid base ratio of the diet

MODIFICATION OF DIET	ANALYSIS			ACID OR BASIC VALUE CALCULATED	NUMBER OF RATS	TIME ON DIET	FRESH THYROID WEIGHT	IODINE IN THYROID (DRY BASIS)
	Ca	P	Ca/P					
	per cent	*per cent*		*per 100 gm. diet*		*days*	*milligrams per 100 gm. body weight*	*per cent*
None	1.51	0.35	4.3	530 cc. 0.1 N Alk.	17	35	48.6	0.0115
$CaCl_2$ instead of $CaCO_3$	1.48	0.31	4.8	....	4	35	18.1	0.0905
$MgCO_3$ instead of $CaCO_3$	0.11	0.38	0.3	....	3	35	24.6	0.0282
10.45% $Na_3PO_4 12H_2O$ added	1.40	1.43	1.0	859 cc. 0.1 N Alk.	4	35	30.2	0.0197
3.7% $NaH_2PO_4.H_2O$ added	1.38	0.98	1.4	311 cc. 0.1 N Alk.	4	35	40.4	0.0098
1.85 cc. H_3PO_4 (85%) added	1.57	0.98	1.6	37 cc. 0.1 N Alk.	4	35	49.7	0.0098
1.85 cc. H_3PO_4 (85%) 2.9 cc. HCl (36.5%)	1.58	1.23	1.3	313 cc. 0.1 N acid	4	35	53.8	0.0162

Basal diet: Yellow corn, 76; wheat gluten, 20; sodium chloride, 1; calcium carbonate, 3; plus 2 drops per rat per day of a diluted solution of oscodal.

and the acid: base ratio of the diet, and, to provide vitamin D, each rat received daily 2 drops of a diluted potent cod liver oil concentrate (Oscodal). The make-up of these diets, and the thyroid weights and iodine content of the thyroids, are shown in table 5. At the time this work was done we did not think to look for iodine contamination in chemically pure calcium chloride; when later we found an amount which was several times that contributed by the remainder of the diet,

the results are understood. It also seems to be rational to assume that the smaller glands obtained with magnesium carbonate and tri-sodium phosphate are due to iodine contamination. Unfortunately, too few rats were used in this work to draw firm conclusions, but the results indicate in rather definite manner, that if the iodine intake is kept low enough, large goiters can be produced regardless of whether the calcium content of the diet be high or low, the calcium-phosphorus ratio normal or abnormal, or the diet acid or alkaline in reaction.

TABLE 6

Effect of vitamin D on the development of goiter (date of experiment April, 1933—duration 56 days)

NUMBER OF RATS	DIET	IRRADIATED YEAST	CALCIUM CARBONATE	ADDED IODINE	BONE ASH (DRY BASIS)	GAIN IN WEIGHT	FRESH THYROID WEIGHT	DRY MATTER IN THYROID	IODINE IN THYROID (DRY BASIS)
		per cent	*per cent*		*per cent*	*gm.*	*milligrams per 100 gm. body weight*	*per cent*	*per cent*
9	Rachitic	None	3	None	30.5	30	31.0 ± 1.3	22.6	0.0125
9	Rachitic	None	1	None	46.7	58	33.9 ± 2.7	23.6	0.0088
8	Rachitic	None	3	3 γ per day	30.4	29	13.5 ± 0.3	31.9	0.2340
9	Rachitic	0.2	3	None	46.2	60	29.6 ± 1.3	23.0	0.0080
9	Rachitic	0.1	3	None	48.2	54	28.7 ± 1.8	22.7	0.0085
9	Rachitic	0.05	3	None	47.5	53	27.4 ± 1.1	22.8	0.0120
9	Rachitic	0.02	3	None	49.9	51	31.0 ± 2.0	23.1	0.0117
9	Rachitic	0.01	3	None	49.1	56	30.2 ± 2.0	22.4	0.0097

D. GOITER AND RICKETS

The Steenbock diet is rachitogenic by virtue not only of vitamin D deficiency, but also its high calcium and low phosphorus content. In the first paper of this series, we stated that either goiter or rickets could be developed independently on this diet, depending on whether it were supplemented with vitamin D or with iodine, but no data were presented in support of this point at that time. Table 6 shows the results of an experiment in which bone-ash determinations were made. The rats were maintained on the experimental diets for 8 weeks instead of the customary 5 weeks, in order that the

differences in bone-ash might be more marked. It will be noted:

1. Of the eight groups of rats fed different modifications of the diet, only that receiving added iodine had thyroids of normal size, normal dry matter content, and normal iodine content.

2. Reduction of the $CaCO_3$ in the diet from 3 per cent to 1 per cent resulted in a high bone ash, but did not lessen the severity of goiter.

3. Addition of irradiated yeast, in quantities from 0.2 per cent down to as little as 0.01 per cent of the diet, resulted in a high bone ash but also in enlarged thyroids of low dry matter and iodine content.

This yeast was claimed to have a vitamin D potency fifteen times that of standard cod liver oil, or over 1200 International units per gram. As little, therefore, as 0.01 per cent in the diet, would furnish in excess of 1 unit per day. We used 0.2 per cent in our diet but growth is but little better on 0.2 per cent than on 0.01 per cent, and significant differences in degree of goiter do not exist.

It appears, therefore, that a reduction in the calcium carbonate of the diet from 3 per cent to 1 per cent, would enable us to produce goiter without rickets even if no source of vitamin D were given, and that, above the protective dose, varying the amount of irradiated yeast up to 0.2 per cent, or an estimated vitamin D intake of 25 units per day, did not affect materially thyroid size, dry matter or iodine content, or growth.

SUMMARY

In extension of previous work on an iodine deficient diet, it is shown that in 125 experimental groups comprising 1021 rats, definite correlations exist between size and iodine content, and between size and dry matter content of the thyroid gland.

From the relationship between weight of gland and iodine content, it is deduced that experimental work on the factors which may influence low iodine goiter should be done within

the critical range, i.e., an iodine content in the gland of 0.03 per cent or less, dry basis, this probably corresponding to an iodine intake from known sources of 0.4 to 0.3 γ per rat per day, and an iodine content in the ration of 35 parts per billion or less.

Since all factors of the environment are difficult to control with certainty, it is suggested that experimental results should be evaluated only in light of simultaneous parallel experiments, and then only if the low iodine controls fall within the critical range.

The storage of iodine by young rats during the first 4 weeks of life is not sufficient to prevent goiter when placed for 5 weeks on a low iodine diet.

Varying the calcium content of the ration, its Ca:P ratio, and the presence or absence of vitamin D do not significantly affect the degree of goiter produced.

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THE ANTI-SCORBUTIC POTENCY OF REVERSIBLY OXIDIZED ASCORBIC ACID AND THE OBSERVATION OF AN ENZYME IN BLOOD WHICH REDUCES THE REVERSIBLY OXIDIZED VITAMIN

JOSEPH H. ROE AND GLENN L. BARNUM

Department of Biochemistry, School of Medicine, George Washington University, Washington

ONE FIGURE

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Tillmans, Hirsch and Siebert ('32) have shown that the first oxidation product formed by the action of 2, 6-dichlorophenolindophenol upon decitrated lemon juice has anti-scorbutic activity and that the oxidation of the indophenol-reducing substance is reversible. Hirst and Zilva ('33) found that the reversibly oxidized product obtained by the action of iodine upon pure ascorbic acid prepared from paprika is only slightly less active than ascorbic acid which had not been oxidized. As these reports do not establish a quantitative evaluation of the anti-scorbutic potency of reversibly oxidized ascorbic acid, it seemed of interest to make a quantitative study of the physiological activity of this vitamin in its reversibly oxidized form, when administered orally and parenterally to guinea pigs. The role of this form of vitamin C in the animal body has also been investigated and will receive attention in this report.

EXPERIMENTAL PROCEDURE

Guinea pigs of market stock weighing around 250 to 300 gm. were placed upon a scorbutic diet consisting of the following: ground rolled oats, 65 parts; alfalfa leaf meal (autoclaved),

25 parts; crude casein, 5 parts; irradiated yeast, 3 parts; sodium chloride, 2 parts. This diet, supplemented with cabbage or other anti-scorbutic foods, was fed to the pigs during a short period of adjustment to laboratory conditions. When the animals were observed to make a normal rate of growth, the anti-scorbutic supplements were withdrawn, and graded doses of pure l-ascorbic acid[1] in the reversibly oxidized form were administered daily, upon a 6-day per week basis, for periods of 6 weeks or more. Negative control experiments with guinea pigs upon the basal diet were also carried out. The preparation of the oxidized form of the vitamin for administration was as follows.

Preparation of reversibly oxidized ascorbic acid

Ascorbic acid was dissolved in distilled water in concentrations that permitted the dosage to be around 1 cc. of solution. Bromine water was added until an excess was present as indicated by yellowness of the solution. Excess bromine was then removed by bubbling air through the solution. The clear solution was neutralized by adding $CaCO_3$, and the excess of the latter was removed by centrifugation. By this procedure ascorbic acid is converted quantitatively into its reversibly oxidized form and a clear, neutral solution, suitable for administration either orally or subcutaneously, is obtained.

Determination of reversibly oxidized ascorbic acid

The solution prepared above was assayed by a colorimetric method developed by one of us (Roe, '35). When ascorbic acid in its reduced form is boiled with HCl, furfural is formed. The latter is readily estimated by the aniline acetate method. Reversibly oxidized ascorbic acid does not form furfural under the same conditions, but readily yields furfural when boiled with HCl containing $SnCl_2$. The latter condition was made the basis of a method for determining reversibly oxidized ascorbic acid, which in brief is as follows.

[1] The authors wish to thank Merck and Company, Rahway, New Jersey, for a liberal supply of pure l-ascorbic acid which was used in this investigation.

One cubic centimeter of solution containing about 0.1 mg. of reversibly oxidized ascorbic acid is placed in a test tube and 1 cc. of 30 per cent HCl containing 1 per cent $SnCl_2$ is added. A standard for comparison is prepared by placing in a test tube 1 cc. of a solution containing an amount of xylose that will produce furfural equivalent to 0.1 mg. of ascorbic acid in its reduced form and adding 1 cc. of the HCl-$SnCl_2$ mixture. (Xylose is used as a standard because it keeps indefinitely when dissolved in saturated benzoic acid.) The two tubes are boiled in a water-bath for 10 minutes, being placed in the bath and removed therefrom simultaneously. After cooling the tubes, 2 cc. of glacial acetic acid and 3 cc. of an alcoholic aniline solution, containing 1 part of redistilled aniline to 2 parts of alcohol, are added to each tube. The tubes are shaken thoroughly to mix the contents and comparison is made in a colorimeter after waiting 5 minutes for maximum color development.

In our experiments reversibly oxidized ascorbic acid was prepared and assayed as indicated above each day, and administration was carried out as soon as conveniently possible after analysis of the solution. In each analysis a control test for reduced ascorbic acid was made. This was done by boiling 1 cc. of the vitamin solution with HCl not containing $SnCl_2$. Under such conditions furfural is not formed from reversibly oxidized ascorbic acid and hence no color is obtained when aniline acetate is added. A solution known to be free of reduced ascorbic acid, and whose quantitative composition had been ascertained with a high degree of accuracy, was thus obtained.

This method of preparing and feeding the oxidized ascorbic acid was adopted for several reasons. Bromine was used because complete oxidation was assured and excess bromine is readily removed by aeration. The solution was neutralized with $CaCO_3$ because at the resulting pH the oxidized vitamin remained stable. Analyses of the oxidized ascorbic acid solution 3 hours after preparation did not reveal any losses. The colorimetric method of analysis permitted a very exact determination of the amount of vitamin present in the solution fed.

To get control data, experiments were carried out with the vitamin in its reduced form. A freshly prepared solution of the same ascorbic acid, dissolved in distilled water and neutralized with $CaCO_3$, was administered each day to a group of guinea pigs to obtain data for comparison with the results of feeding the oxidized form.

The dosage of vitamin was based upon body weight in accordance with the method of assay of vitamin C adopted by Dann and Cowgill ('35). The pigs were weighed weekly and the dosage was changed at the time of each weighing. The average weight of each group under study was taken as the basis for the calculation of the new dosage.

TABLE 1

Feeding experiment using ascorbic acid in the reduced and the reversibly oxidized forms

GUINEA PIG NO.	ASCORBIC ACID			WEIGHT		
	Form administered	Method of administration	Dose per 100 gm. of body weight	Initial	6 weeks	8 weeks
			mg.	*gm.*	*gm.*	*gm.*
1	Oxidized	Subcutaneous	0.25	275	255	
2	Oxidized	Subcutaneous	0.25	278	240[1]	
3	Oxidized	Subcutaneous	0.25	265	237	
4	Oxidized	Subcutaneous	0.25	257	182[1]	
5	Reduced	Subcutaneous	0.25	278	458	
6	Reduced	Subcutaneous	0.25	290	462	
7	Reduced	Subcutaneous	0.25	255	388	
8	Reduced	Subcutaneous	0.25	247	401	
9	Oxidized	Subcutaneous	0.50[2]	255	365	413
10	Oxidized	Subcutaneous	0.50	275	363	409
11	Oxidized	Subcutaneous	0.50	262	320	378
12	Oxidized	Subcutaneous	0.50	248	355	418
13	Oxidized	Oral	0.50	265	315	390
14	Oxidized	Oral	0.50	250	374	470
15	Oxidized	Oral	0.50	248	328	385
16	Oxidized	Oral	0.50	268	378	455

[1] Guinea pigs nos. 2 and 4 died 2 and 4 days, respectively, before the end of the 6-week period.

[2] Five-tenth milligram per 100 gm. was administered to pigs 9 to 16, inclusively, for 6 weeks and then the dose was raised to 1 mg. per 100 gm.

Results of feeding experiments

The results of these experiments are shown in table 1 and figure 1. When 0.25 mg. of reversibly oxidized ascorbic acid per 100 gm. of weight was injected subcutaneously into four guinea pigs daily, the animals lost weight and showed some clinical signs of scurvy. Two pigs survived for 6 weeks upon this regime and the other two died during the fifth week.

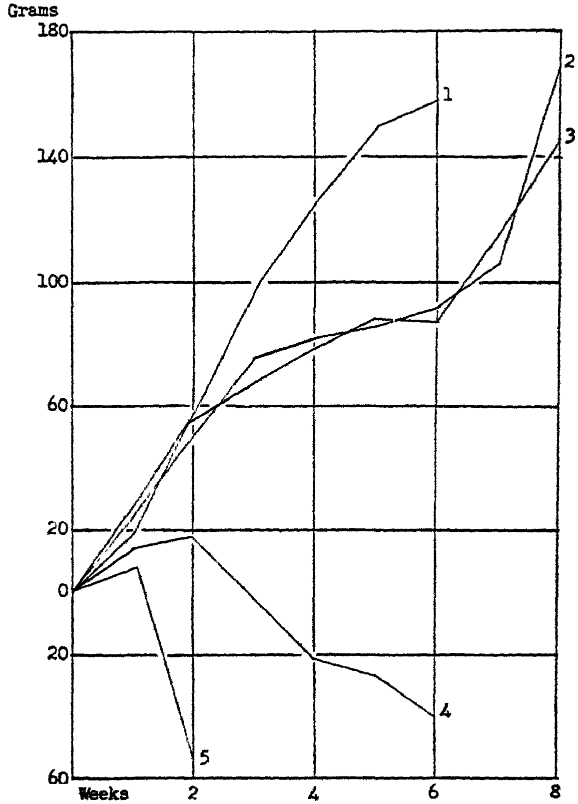

Fig. 1 Composite growth curves showing the cumulative weekly gains of guinea pigs receiving daily doses of ascorbic acid per 100 gm. of body weight as follows: 1, 0.25 mg. of l-ascorbic acid in its reduced form, subcutaneously; 2, 0.5 mg. of reversibly oxidized l-ascorbic acid for 6 weeks, and 1 mg. of the same preparation for the last 2 weeks, orally; 3, same as 2 except that the oxidized vitamin was administered subcutaneously; 4, 0.25 mg. of reversibly oxidized l-ascorbic acid, subcutaneously; 5, negative controls with basal diet only.

Four guinea pigs upon the same diet receiving the same dosage of ascorbic acid in the reduced form by subcutaneous injection grew at a normal rate and remained free from symptoms of scurvy. These experiments showed that the reversibly oxidized form of ascorbic acid has protective properties, but that its potency is considerably less than that of the vitamin in its reduced form.

A dosage of 0.5 mg. of reversibly oxidized ascorbic acid per 100 gm. of weight was next used. The vitamin was administered to eight guinea pigs, four being fed orally and four receiving the ascorbic acid by subcutaneous injection. These animals gained in weight and remained free from clinical symptoms of scurvy for 6 weeks. The rate of growth was not rapid, however, upon this amount of vitamin, and the dosage was therefore raised to 1 mg. per 100 gm. of tissue. Upon this dosage of the reversibly oxidized ascorbic acid the pigs grew at a more rapid rate, the gain in weight apparently being maximum.

Control guinea pigs upon the same diet as used in the above experiments, but not receiving vitamin supplements, lost weight rapidly, as shown in figure 1, and died in about 3 weeks from scurvy which was obvious both clinically and at autopsy.

Some of the animals in the group receiving 0.5 mg. and 1 mg. per 100 gm. of tissue were autopsied 48 hours after the oxidized vitamin supplements had been discontinued. Slight hemorrhages in the knee joints, brittleness of the bones and teeth, and other evidences of beginning scurvy were observed in those pigs which had received the vitamin supplement parenterally. The pigs which had been fed the oxidized ascorbic acid by mouth did not show any signs of scurvy.

Chemical analyses for both oxidized and reduced ascorbic acid were carried out upon the liver, muscle, brain, and adrenal glands of the animals receiving 0.5 mg. and 1 mg. of the oxidized vitamin per 100 gm. of tissue. Our colorimetric method was used to determine both the oxidized and reduced vitamin C content and the analyses for the reduced vitamin content were checked with the Tillmans' 2, 6-dichlorophenol-indophenol method, as modified by Bessey and King ('33).

The results of these chemical analyses showed that there was practically no vitamin C stored in the tissues of the animals receiving reversibly oxidized ascorbic acid, either orally or by subcutaneous administration, the data obtained in these studies being essentially within the blank values of the methods used.

Reductase in blood

In view of these findings it became desirable to study the relation of reversibly oxidized ascorbic acid to metabolism. It was obvious that either the oxidized form is an intermediate in the metabolism of reduced ascorbic acid or else the oxidized vitamin is converted into its reduced form by the tissues. The first assumption seemed highly improbable since it was found that the oxidized form has a much lower potency than the reduced form. The second hypothesis seemed more likely and experiments were planned to determine its validity.

Blood was used to study the ability of tissues to reduce oxidized ascorbic acid because this tissue maintains a more normal metabolism after removal from its natural environment than other tissues obtained by slicing or maceration. Ascorbic acid, dissolved in 0.9 per cent NaCl solution, was oxidized with bromine and the solution was neutralized with $CaCO_3$, the procedure being entirely similar to that used to prepare the oxidized vitamin in the feeding experiments. Potassium oxalate was used as an anticoagulant for the blood and pooled samples of human blood were used for most of the experiments. Whole blood, blood plasma, and washed red cells were mixed with oxidized ascorbic acid and placed in an incubator at 38°C. for 3 hours. At the end of the incubation period the blood mixtures which contained red cells were centrifuged. Five cubic centimeter portions of the plasma, or the centrifugate from the red cells, were diluted with 20 cc. of water, 1 cc. of glacial acetic acid was added, and the mixture was titrated with 2, 6-dichlorophenolindophenol.

The results of these experiments are shown in table 2, which includes representative data from a larger series. Human

blood, guinea pig blood, and rat blood were found to have the capacity to reduce reversibly oxidized ascorbic acid. In experiments 1 and 2, human plasma and serum gave characteristic indophenol reduction values after incubation at 38°C. with oxidized ascorbic acid for 3 hours. In experiment 3 human red cells were washed ten times with 0.9 per cent NaCl

TABLE 2

Effect of blood on reversibly oxidized ascorbic acid. Indophenol titration of oxalated blood after 3 hours incubation at 38°C. with 1 mg. of reversibly oxidized ascorbic acid per cubic centimeter. The titrations were made upon 5 cc. of fluid, except in experiment no. 9

EXPERIMENT	2, 6-DICHLOROPHENOLINDOPHENOL TITRATION		
	Total	Blank	Amount due to reduced form of the vitamin
	cc.	*cc.*	*cc.*
1. Human plasma	13.4	2.8	10.6
2. Human serum	8.8	1.8	7.0
3. Centrifugate from human erythrocytes washed ten times with 0.9 per cent NaCl solution	10.1	2.8	7.3
4. Human plasma plus 1 mg. NaF per cubic centimeter	10.9	2.9	8.0
5. Human plasma plus 2 mg. NaF per cubic centimeter	2.9	2.9	0
6. Human plasma plus 5 mg. NaF per cubic centimeter	2.9	2.9	0
7. Human plasma, control on 4, 5 and 6	13.0	2.9	10.1
8. Human plasma, heated 5 minutes at 100°C.	3.4	3.4	0
9. 50 cc. tungstic acid filtrate, 1:10 dil.	1.5	1.0	0.5
10. Human plasma plus 1 cc. $CHCl_3$	14.5	3.4	11.1
11. Human plasma control on 10	16.8	3.4	13.4
12. Human plasma plus 1 cc. ethyl ether	11.3	1.8	9.5
13. Human plasma control on 12	11.2	1.8	9.4
14. Guinea pig plasma	14.0	2.5	11.5
15. Rat blood, erythrocyte centrifugate, 0.9 per cent NaCl	9.0	2.5	6.5

solution and then physiological salt solution containing oxidized ascorbic acid was added in a quantity equal to the amount of plasma removed. After 3 hours incubation at 38°C. the red cell centrifugate showed a characteristic reduction of indophenol.

Experiments were carried out to determine whether the substance in blood which reduces oxidized ascorbic acid is

an enzyme. Plasma was mixed with sodium fluoride, chloroform, and ethyl ether. Sodium fluoride in a concentration of 2 mg. per cubic centimeter of plasma completely inhibited the reduction of oxidized ascorbic acid. Chloroform only interfered slightly and ether had no effect upon the reduction. After plasma had been heated for 5 minutes on a water-bath at 100°C. it did not show any capacity to reduce the oxidized vitamin. Obviously heating coagulates the proteins of blood plasma and does violent damage to the system, but a heating experiment was performed since destruction by heat is one of the criteria to determine the presence of an enzyme. The removal of proteins from whole blood with tungstic acid gave a filtrate with a negative capacity to reduce oxidized ascorbic acid.

The above results show that there is a substance in blood which has the ability to reduce oxidized ascorbic acid and they seem to indicate clearly that this substance is an enzyme.

Experiments with urine gave entirely negative results. There is no substance in urine having the power to reduce oxidized ascorbic acid under the conditions of our experiments with blood.

DISCUSSION

The data of this report show that ascorbic acid is approximately one-fourth as potent in its oxidized form as in its reduced form when judged by the promotion of growth and the prevention of clinical signs of scurvy in the guinea pig. Oral administration of the reversibly oxidized vitamin is much more effective in preventing the onset of scurvy than is the parenteral method. The pigs given the oxidized vitamin by subcutaneous injection showed beginning signs of scurvy in 48 hours after withdrawal of the vitamin, while the pigs given the oxidized vitamin by mouth, autopsied 1 week after discontinuance of the vitamin, did not show signs of scurvy. These results are opposite to those observed by Grollman and Firor ('34) and Hou ('35) with the vitamin in its reduced form, the latter authors finding the reduced form of vitamin C more potent when administered parenterally. The greater

protection of the oxidized vitamin, when administered by mouth, is believed to be due to the more delayed introduction of the vitamin into the tissues. In an experiment in which reversibly oxidized ascorbic acid was mixed with intestinal contents of a guinea pig and placed at 38°C. it could not be demonstrated by indophenol titration that the oxidized vitamin is reduced by intestinal contents.

The oxidized vitamin fed by us is apparently the first oxidation product of ascorbic acid. When assayed by our colorimetric method of analysis, it was found to be quantitatively converted into the reduced form of ascorbic acid by HI, and $HCl\text{-}SnCl_2$, and by treatment with H_2S 80 per cent is returned to the reduced form. The product has an $[\alpha]_D^{27}$ value of $+ 52.5°$ in acid solution with a pH of 0.90.

The results obtained by us are not in quantitative agreement with the report of Hirst and Zilva ('33), who found that oxidized ascorbic acid "is only slightly less active than ascorbic acid which had not been previously oxidized." Hirst and Zilva prepared their compound by oxidation with iodine, while our preparation was an oxidation product of bromine, but it is believed our compound is the same as that fed by Hirst and Zilva, as the regeneration data and specific rotations of the two compounds are in fair agreement.

The explanation of the anti-scorbutic activity of oxidized ascorbic acid is undoubtedly that this compound is converted into its reduced form by a substance present in the blood. This substance is believed to be an enzyme because it is inactivated by sodium fluoride, heating, and deproteinization with tungstic acid.

The finding of a reductase for oxidized ascorbic acid in blood is of considerable interest. From this observation it seems probable that ascorbic acid exists in the blood only in the reduced form. This enzyme may perhaps have functions other than maintaining vitamin C in its reduced form and might participate in other ways in the oxidation-reduction system of the animal body.

SUMMARY

1. Data have been obtained which show that reversibly oxidized ascorbic acid has approximately one-fourth the anti-scorbutic potency of ascorbic acid in its reduced form when administered to guinea pigs.

2. Reversibly oxidized ascorbic acid is more potent anti-scorbutically when given orally than when administered subcutaneously.

3. Ascorbic acid is not stored in the tissues of guinea pigs, either in the oxidized or reduced form, when the reversibly oxidized form is administered in amounts as high as 1 mg. per 100 gm. of body weight per day.

4. The anti-scorbutic effect of reversibly oxidized ascorbic acid is due to an enzyme in blood which has the power to reduce the reversibly oxidized form of this vitamin.

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RESULTS OF FEEDING VARIOUS LEVELS OF SOIL CONTAINING BERYLLIUM TO CHICKENS, DOGS AND RATS[1]

C. W. DUNCAN AND E. J. MILLER

Section of Chemistry, Michigan Agricultural Experiment Station, East Lansing

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The experimental work reported in this paper was originally initiated for the purpose of investigating the report that the ingestion of soil by animals in certain counties in northern Michigan was deleterious to their health and well-being. The toxicity of the soil was thought to be due to its beryllium content. The results are of additional interest because of the absence in the literature of data on the effect of ingestion of soil by animals.

Guyatt, Kay and Branion ('33) demonstrated that 'beryllium rickets' was produced in rats which received a normal diet by the addition of small amounts of beryllium carbonate. The diet contained optimum amounts of calcium and phosphorus and a generous allowance of cod liver oil. Jacobson ('33) found that the Steenbock diet 2965 modified by the substitution of a chemically equivalent amount of beryllium carbonate for calcium carbonate produced a disease in rats which was anatomically indistinguishable from rickets. Fabroni ('33), however, stated that the intravenous injection of beryllium hydroxide into rabbits had no demonstrable toxic or harmful effect despite the storage of the colloid in the reticulo-endothelial system. Sobel, Goldfarb and Kramer ('35) eliminated the factor of intestinal absorption by studying the mechanism of calcification under in vitro conditions

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and concluded that there was a local disturbance in calcification in beryllium rickets.

In view of the above reports in the literature concerning the toxicity of beryllium, feeding experiments were conducted to ascertain the possibility of the existence of a natural beryllium toxicosis and to observe also the effect of feeding high levels of soil to various animals. For purposes of comparison some of the animals were fed beryllium carbonate with the control ration.

EXPERIMENTAL

Samples of the suspected soil were analyzed qualitatively by the method of Chamot and Mason ('31), quantitatively by the method of Washington ('30), and spectrophotometrically.

The mechanical analysis of the soil, which was of the silty loam type, showed that it contained 54.0 per cent fine sand, 21.6 per cent silt and 24.4 per cent clay. Approximately 33 per cent of the clay was colloidal.

The mean value found from ten quantitative analyses of the suspected soil was 0.615 per cent beryllium oxide, which was equivalent to 0.223 per cent beryllium. Martin ('33) stated that the United States Bureau of Mines had found comparable amounts of beryllium in the soil and that the beryllium was in the form of beryl. Beryllium was also detected in samples of soil from the Upper Peninsula, the College Farm and a sample of the Hillsdale type of soil.

The rat stock ration was composed of:

	parts		*parts*
Yellow corn	36	Alfalfa	5
Barley	30	Bone meal	2
Skim milk powder	10	Salt	1
Blood meal	5	Cod liver oil	1

The chemical analysis of the above ration was as follows:

	per cent		*per cent*
Moisture	9.30	Calcium	0.92
Protein	18.24	Phosphorus	0.67
Ash	5.71	Magnesium	0.16
Crude fiber	3.93	Fat	3.49

The blood was obtained from the chickens by heart puncture and pooled for each group. The blood from the dogs was also obtained by heart puncture. Lithium citrate was used as the anticoagulant. The plasma calcium and inorganic phosphorus values were determined by the Shohl ('22) modification of the Kramer-Tisdall method and the Briggs ('22) modification of the Bell-Doisy technic, respectively.

The right tibia of each chicken and the right humerus of each dog were cleaned, sawed into desirable lengths and extracted with hot 95 per cent alcohol for 72 hours. The fat-free, moisture-free bones were weighed into crucibles and ashed. After cooling in a desiccator the samples were reweighed and ground to pass a 20-mesh sieve. Aliquot portions of the ash were taken for the determination of calcium and phosphorus by the above-mentioned methods and the magnesium by a modification of the Briggs technic (Duncan, Huffman and Robinson, '35).

Rat feeding trials. Three groups of albino rats, each containing four animals approximately 28 days of age, were fed the regular stock rat ration which had been supplemented with, 1) 10 per cent of the soil, dried and ground to pass a 40-mesh sieve, 2) 20 per cent of the soil and 3) 0.25 per cent beryllium carbonate. The rats in groups 1 and 2 tolerated the soil well, grew normally and seemed to thrive on the diet. The rats in group 3 began to show rachitic symptoms within 30 days. Their gait became waddling, their backs were arched and the ankle joints were enlarged and stiff. The rats were definitely under normal weight and all of the rats in this group manifested rickets. The rats in the other two groups, however, were normal in weight and in appearance. After 40 days on experiment, the rats in groups 2 and 3 were killed and subjected to macroscopic examination. The rats in group 3 showed the typical rachitic symptoms. There were also indications of slight anemia. None of the symptoms observed in the rats rendered rachitic by beryllium carbonate were observed in the rats which had had 20 per cent of their diet supplemented with soil.

The soil intake was then increased to 20 per cent of the diet of the rats in group 1 with the object of studying the effect of feeding a high level of soil over a long period of time. The rats were allowed to mature on this diet, breed and reproduce. No changes were made in the diet of the pregnant or lactating mother except to add occasionally a few drops of cod liver oil. The litters were always reduced to six. After each of the female rats had produced one litter and had raised them to weaning age, the mothers and the male were destroyed. All of the offspring were kept on the experimental diet for 30 days after weaning. At that time, six female and one male rat were selected to be continued on experiment. The rats which were destroyed were always examined for rachitic symptoms. In the above manner, four successive generations of rats were raised on the regular stock diet supplemented with 20 per cent soil. The diet of the fifth generation was modified by increasing the soil intake to 30 per cent. Three generations of rats were raised on the high soil level. All of the rats were normal in every respect; they grew well and seemed to thrive on the diet.

The amount of beryllium in the 20 and 30 per cent soil diets was equivalent to 0.0446 and 0.0669 per cent, respectively, whereas the amount of beryllium in the 0.25 per cent beryllium carbonate diet was equivalent to 0.0329 per cent.

Chicken feeding trials. Eighty chickens, about 8 weeks of age, were divided into four approximately equal groups with respect to weight. Each group was fed a standard growing mash plus 2 per cent cod liver oil to compensate for laboratory conditions. In addition to the above ration, pen A had 5 per cent of the ration supplemented with soil which had been dried and ground to pass a 40-mesh sieve. Pens B and C each had 10 per cent of the ration supplemented with suspected soil from two sources. Pen D was the check group. The chickens were weighed at the beginning of the experiment and at regular intervals thereafter. The average percentage gains in weight of each group after being on experiment for 3 weeks are shown in table 1.

TABLE 1

Average initial weights and gains in weight of the chickens after 3 weeks on experiment

	INITIAL WEIGHT	FINAL WEIGHT	GAIN WEIGHT	GAIN
	gm.	*gm.*	*gm.*	*per cent*
Pen A	347	609	262	75.5
Pen B	335	580	245	73.2
Pen C	365	650	285	78.1
Pen D	411	670	259	63.0

The results of this phase of the experiment did not indicate any deleterious effects from the ingestion of soil so chickens in pen D were discarded and the rations were modified to increase the levels of soil intake. The chickens in pen A were designated pen E and the soil intake was increased to 25 per cent. The chickens in pens B and C received the same amount of soil as before but additional soil was kept before them at all times. Three chickens each from pens B and C were put into a separate pen and fed the soil as 50 per cent of the ration. These chickens were designated pen F. The average rate of growth of the chickens in each group is presented in table 2.

TABLE 2

Average weight of the chickens at the end of each period

	INITIAL WEIGHT	WEIGHT 3RD WEEK	WEIGHT 5TH WEEK	WEIGHT 7TH WEEK	WEIGHT 11TH WEEK	WEIGHT 3.5 MONTHS
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Pen B	335	580	813	991	1471	2178
Pen C	365	650	885	1012	1464	2018
Pen E	347	609	866	1018	1552	2262
Pen F		435	685	892	1353	1935

The most interesting observation that can be made from a study of table 2 is that the chickens which received a ration supplemented with 25 per cent soil made the largest gains, whereas the chickens which had 50 per cent of their ration supplemented with soil made almost as good gains. The chickens which had 10 per cent of their ration supplemented with soil and received soil ad lib. made intermediate but comparable gains.

The chickens were sacrificed after being on experiment for 3.5 months and the blood and bones were analyzed for their mineral content. The results are composited and summarized in table 3.

TABLE 3

Composition of bones and blood of chickens receiving various levels of soil (composite)

	RIGHT TIBIA				BLOOD PLASMA	
	Ash	Ca	P	Mg	Ca	P
	per cent	*per cent*	*per cent*	*per cent*	*milligrams per 100 cc.*	
10 per cent soil + soil ad lib.	58.00	22.35	10.10	0.60	12.3	6.68
10 per cent soil + soil ad lib.	58.40	22.13	10.26	0.83	9.1	7.23
25 per cent soil	55.35	21.43	9.64	0.59	11.2	6.72
50 per cent soil	54.50	20.81	9.55	0.77	11.5	6.88

The results from this experiment indicate that the feeding of soil to young chickens did not produce any observable ill effects due to the beryllium content or to the high levels of soil intake. Growth was not interfered with nor was the percentage composition of the tibia reduced because of the high intakes of soil.

Dog feeding trials. Since foxes were among the animals supposed to be affected by the soil, it was deemed advisable to test the soil on dogs. It was thought also that dogs would demonstrate any toxicosis due to the ingestion of soil better than any other species of animals because of the high hydrochloric acid content of their gastric secretions.

Four puppies which had been raised under the same conditions in regard to diet and general surroundings were brought to the laboratory and placed in a large cage immediately after weaning. The puppies were of mongrel breed, two being males and two females. Each puppy received a mixture of a commercially prepared dog meat and commercially prepared dog biscuit in amounts according to the needs of each animal. They also received 1 pint of milk and 1.0 cc. of cod liver oil each per day. Each puppy was fed in an individual cage. Dog 1 was the control and received the above unsupplemented ration. Thirty per cent of the ration, on the dry-matter basis, of dog 2 was supplemented with soil

which had been dried and ground to pass a 40-mesh sieve. Sixty per cent of the ration of dog 3 was supplemented with soil. The ration of dog 4 was supplemented with 0.75 gm. of beryllium carbonate, equivalent to 0.10 gm. of beryllium, per day, throughout the experiment. In the ration which contained 30 per cent soil, the actual beryllium intake varied from 0.045 gm. per day at the beginning of the experiment to 0.08 gm. per day when the experiment was completed. The 60 per cent soil ration varied from 0.09 gm. to 0.15 gm. per day of beryllium.

After about 15 days on experiment, dog 4 began to refuse the dog biscuits and after 30 days, the biscuits were refused at all times. At about this time dog 4 also began to refuse cold milk but when the milk was warmed to room temperature, it was readily consumed. The dog acted as though its mouth and teeth were sore and upon examination it was found that the teeth were less firmly embedded than normal. Dogs 1, 2 and 3 had ravenous appetites at all times. On several occasions, however, dog 3 would be off-feed in the morning or evening but would make up for it at the next feeding. The feeding experiments were continued for 3 months.

Table 4 shows the weight of each dog when placed on experiment and the rate of growth in body weight throughout the feeding trials.

TABLE 4

Changes in body weight of the four dogs

DATE	DOG 1	DOG 2	DOG 3	DOG 4
1934	*gm.*	*gm.*	*gm.*	*gm.*
6–19	1080	1625	1380	1690
6–26	1360	2130	1800	1955
7–11	1965	2900	2320	2130
7–25	2960	4180	3280	2210
8–10	3940	5290	4170	2110
8–25	4980	6845	5370	2090
9–17	6370	8195	6825	1880

The physical condition of dogs 1, 2 and 3 at the completion of the experiment was, to all outward appearances, good.

Their coats of hair were sleek, the teeth were normal and well developed, their legs were strong and straight and they were alert and playful. There were no evidences of rickets, abnormal cravings for foreign objects or physical deformities. Dog 4, however, grew slowly during the first 5 weeks on experiment and then progressively lost weight. The ankle joints became enlarged and the back became arched. The dog showed extreme emaciation in spite of the fact that the appetite for soft food was good. The dog was almost unable to stand unassisted at the termination of the experiment.

Table 5 presents some of the physical measurements of three dogs at the end of the experiment.

TABLE 5

Physical measurements of the experimental dogs

	DOG 1	DOG 3	DOG 4
	cm.	*cm.*	*cm.*
Length of head and neck	14	16	15
Length of body	42	43	28
Length of tail	23	25	16
Length of front legs	17	17	12
Height at front shoulders	39	41	20
Height at rear quarters	36	39	24

The data in table 5 demonstrate the stunting effect upon the growth of dog 4 due to the ingestion of beryllium carbonate. This effect was not noted in dog 3 which was fed the soil as 60 per cent of the ration and equivalent to 0.09 to 0.15 gm. of beryllium per day.

Table 6 gives the concentrations of calcium and inorganic phosphorus in the blood plasma and the ash and mineral content of the right humerus of each dog.

TABLE 6

Concentrations of blood plasma calcium and inorganic phosphorus and the ash and mineral content of the humeri

	BLOOD PLASMA		RIGHT HUMERUS			
	Ca	P	Ash	Ca	P	Mg
	milligrams per 100 cc.		*per cent*	*per cent*	*per cent*	*per cent*
Dog 1	13.1	4.77	56.45	21.22	10.02	0.84
Dog 3	13.1	8.23	54.45	20.90	9.62	1.26
Dog 4	12.6	2.44	55.40	21.93	9.46	0.55

It will be noted that the plasma calcium and inorganic phosphorus were normal in dogs 1 and 3. The calcium was normal in dog 4 but the inorganic phosphorus was markedly subnormal. The ash and mineral content of the humeri of all of the dogs were normal and showed good agreement. It is well known that growth is an important factor in the development of rickets, the greater the rate of growth, the more marked the tendency to develop rickets in animals. The lack of growth works strongly against the induction of rickets and is well illustrated in the ash and mineral content of the humerus of dog 4.

Post-mortem examination was made of each dog. Dogs 1 and 3 were in good condition of flesh and moderately fat. All of the internal organs were normal in color. There was no evidence of anemia. Dog 4 was extremely emaciated and the internal organs were lighter in color than normal because of slight anemia. The heart and gall bladder were unusually large for the size of the dog. There was one yellow gelatinous mass, about 1 cm. in diameter, on the liver. The ribs showed marked rachitic beading. There were no evidences of decayed or malnourished teeth, although the dog's mouth had been sensitive to hard food and cold liquids.

DISCUSSION

Seven successive generations of rats were raised which had their diet supplemented with the suspected soil. The first four generations had 20 per cent of their diet replaced by the soil which was equivalent to the ingestion of 0.0446 per cent beryllium. The diet of the fifth generation was increased to 30 per cent and the two succeeding generations were raised to maturity on the higher level. The ingestion of this amount of soil was equivalent to 0.0669 per cent beryllium. The ingestion of a small amount of beryllium carbonate (0.0329 per cent beryllium) with an otherwise adequate diet resulted in the production of rachitic symptoms in rats in 20 to 30 days.

The young rats were placed on the experimental diet at weaning age because young rats are more susceptible to a rachitogenic regime. This procedure proved ineffective since the beryllium contained in the soil was unavailable to the animal body. The results from long-time observations with rats indicate, however, that these animals can tolerate unusually large amounts of soil in their diet.

The experiment with young chickens did not indicate any deleterious effects when the soil was fed up to 50 per cent of the ration. Growth was not interfered with and the bone ash determinations did not indicate poor utilization of calcium and phosphorus due to the ingestion of large amounts of soil.

The experiment with dogs indicated that intakes of soil up to 60 per cent of the ration were well tolerated. The dog on the high soil ration refused his feed on several occasions but this did not result in poor growth, a mineral deficiency or malnutrition. There were no unfavorable results due to the beryllium content or to the high levels of soil intake. The dog which received a small amount of beryllium carbonate soon developed all of the outward symptoms of rickets.

One of the chemical properties of beryllium is the formation of a phosphate which is exceedingly insoluble, even in moderately acid solutions. Any ingested soluble beryllium salt would be decomposed and the beryllium quantitatively precipitated as the phosphate by any free phosphate present in the intestinal tract. This insoluble precipitate would be excreted and thereby cause a constant drain on the animals' intake of phosphorus. In view of the fact that the beryllium compound present in the soil is extremely insoluble in all ordinary reagents, it was, therefore, impossible to get enough beryllium into solution in the gastric or intestinal secretions of the experimental animals to cause a serious phosphorus deficiency.

These experiments failed to produce any bone lesions which could be associated with rickets or to the ingestion of a radioactive substance. These results do not substantiate the belief of Hanna ('33) that beryllium has distinct radioactive properties.

Evvard and associates ('27) found that the feeding of black loam soil to 2-year-old feeder steers resulted in greater body gains, a lower cost of gain and considerably more internal fat than for a corresponding lot of steers which received the same basal ration minus the soil.

The results of these experiments also confirm the finding of Weinert ('35) who reported that the feeding of quartz sand up to 31.5 per cent of the ration had no marked influence on the digestive function of wethers, that sand in amounts up to 30 per cent or dirt up to 25 per cent of the ration had no marked influence upon milk production in cows over 10-day periods, and that a diet containing 50 per cent sand did not affect the appetite or the digestive function of growing rats.

SUMMARY AND CONCLUSIONS

Feeding experiments with rats, chickens and dogs have been conducted to investigate the possibility of the occurrence of a natural beryllium toxicosis due to the ingestion of soil.

The feeding of a diet which contained 30 per cent soil to rats was well tolerated and did not predispose them to any of the rachitic symptoms produced in the rats which received 0.25 per cent beryllium carbonate in their diet.

Chickens tolerated up to 50 per cent of the soil in their ration. The beryllium in the soil failed to interfere with normal growth or to reduce the concentration of inorganic phosphorus in the blood plasma.

The ingestion of a ration which contained 60 per cent soil was well tolerated and did not result in poor growth in dogs, nor did it produce a phosphorus deficiency due to the beryllium in the soil.

These results fail to verify the report that the ingestion of soil in certain counties in northern Michigan produced malnourished and deformed animals. The amount of soil fed to the animals in these experiments exceeded any amount that the animal would naturally consume when receiving a well-balanced ration.

We are indebted to Dr. Frances Lamb for spectrophotometric determinations and to Dr. G. J. Bouyoucos for the mechanical analysis of the soil.

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THE VITAMIN CONTENT OF CANNED PINEAPPLE JUICE

N. B. GUERRANT, R. ADAMS DUTCHER, FLORENCE S. TABOR AND RUSSEL RASMUSSEN

Department of Agricultural and Biological Chemistry, Pennsylvania State College

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INTRODUCTION

The juices of certain fruits and vegetables have become exceedingly popular as constituents of the human dietary. This increased popularity is apparently due to a combination of qualities or factors, such as palatability, nutritional value, ease of serving, etc. A juice which has received favorable consideration during the past year is that of the pineapple. As to the nutritive value of commercially canned pineapple juice, especially concerning its vitamin content, little can be said, because of the lack of definite scientific data. Data are available, however, which show that both fresh and canned pineapple are good sources of several of the vitamins and, in addition, contain other highly desirable nutritive properties. The investigations of Miller ('24) and Killian ('32) are referred to in this connection.

Since the data presented by the above investigators indicate that both fresh and canned pineapple are good sources of several of the vitamins, it seemed that similar data concerning the vitamin content of commercially canned pineapple juice would have a definite value at this time. The studies herein reported were carried out for the purpose of determining the vitamin A, B, C and G content of this juice.

EXPERIMENTAL

Vitamin A. The method used in the determination of the vitamin A potency of the pineapple juice was essentially that of Sherman and Munsell ('25). Young rats, 20 to 21 days of age and weighing between 40 and 46 gm., were used as the experimental animals. At weaning, each rat was placed in an individual metal cage having a raised screen floor, and was supplied with fresh distilled water and a liberal quantity of the basal (vitamin A-free) diet. An allotment of 0.6 gm. of dry irradiated baker's yeast was fed daily as a source of vitamins B, D and G. All animals were weighed at frequent intervals, at which time a record was made as to the amount of food consumed and the general well-being of the animals. When the animals had been depleted of their body stores of vitamin A, as indicated by cessation of growth and the appearance of incipient xerophthalmia, they were arranged in groups of from six to ten animals, at which point each animal was fed daily an accurately measured quantity of the pineapple juice as the supplement to the vitamin A-free diet. In every case, this supplementary feeding of pineapple juice was continued for a period of at least 6 weeks before the experiments were terminated.

The results obtained through this series of experiments are presented in condensed form in table 1.

The data presented in table 1 indicate that 1 ml. of this juice contains approximately 1 Sherman unit of vitamin A. On the basis of this assay, therefore, the canned pineapple juice tested contained approximately 30 Sherman units of vitamin A per ounce.

Vitamin B (B_1). The Chase and Sherman ('31) method was used in determining the vitamin B potency of the canned pineapple juice. The experimental animals and their care and management were essentially the same as that employed in the vitamin A assays, excepting that a vitamin B deficient diet was employed. When the animals had been depleted of their body reserves, as indicated by cessation of growth and the appearance of mild symptoms of paralysis, they were

arranged in groups of from six to ten animals each. A measured amount of the juice to be tested was fed daily in a separate receptacle to each animal. The feeding of the juice was continued for a period of 6 weeks, during which time an accurate record was kept of each animal's weight, the amount of food consumed, and the general appearance of the animal.

TABLE 1

Showing the effect on growth of feeding different daily allotments of pineapple juice to groups of rats which were receiving a diet deficient in vitamin A. For sake of comparison a similar group of rats was fed 1 γ of International carotene daily as a source of vitamin A

DEPLETION PERIOD							SOURCE OF VITAMIN A. QUANTITY FED DAILY	CURATIVE PERIOD						
		Average weight at end of						Average weight at end of						
Group	Average initial weight	7 days	14 days	21 days	28 days	Depletion period		7 days	14 days	21 days	28 days	35 days	42 days	Gain in weight
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	42	64	81	107	123	129	None	127	122	115	...	...	...	..
2	45	70	86	106	134	138	1.0 ml. of pineapple juice	147	154	159	159	159	161	23
3	44	68	85	100	126	132	1.5 ml. of pineapple juice	142	147	154	158	161	162	30
4	44	68	91	116	130	135	2.0 ml. of pine apple juice	143	149	158	163	165	169	34
5	45	67	89	111	131	134	3.0 ml. of pineapple juice	143	150	159	165	169	176	42
6	44	68	87	116	131	138	4.0 ml. of pineapple juice	149	161	172	181	191	196	58
7	45	67	93	114	132	136	5.0 ml. of pineapple juice	143	155	169	180	187	198	62
8	41	61	80	103	118	123	1 γ International carotene	130	139	143	149	153	158	35

The results obtained in this series of experiments are presented in condensed form in table 2.

From these results it is evident that 1.5 ml. of this canned pineapple juice contained at least 1 Sherman unit of vitamin B. On this basis, 1 ounce of this juice contained approximately 20 Sherman units of this vitamin.

Vitamin G (B_2). The method used in determining the vitamin G potency of the canned pineapple juice was that

described by Bourquin and Sherman ('31). Here again young rats, 20 to 21 days of age and weighing between 40 and 46 gm., were used as the experimental animals. The care and treatment of the experimental animals was the same as that reported under vitamin A. After the test animals had been partially depleted of their body stores of vitamin G,

TABLE 2

Showing the growth stimulating effect of pineapple juice when fed at different levels to groups of rats which were receiving a diet deficient in vitamin B. For sake of comparison a similar group of rats received 10 mg. of an International vitamin B preparation

DEPLETION PERIOD					SOURCE OF VITAMIN B. QUANTITY FED DAILY	CURATIVE PERIOD						
		Average weight at end of				Average weight at end of						
Group	Average initial weight	7 days	14 days	Depletion period		7 days	14 days	21 days	28 days	35 days	42 days	Gain in weight
	gm.	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	42	53	58	64	None	60	49	40	..	..	..	..
2	45	59	64	66	1.0 ml. of pineapple juice	68	69	70	72	73	70	4
3	42	56	63	62	1.5 ml. of pineapple juice	68	71	73	75	81	85	23
4	45	55	65	64	2.0 ml. of pineapple juice	67	74	80	85	89	90	26
5	42	52	63	60	3.0 ml. of pineapple juice	64	76	82	84	92	96	36
6	43	53	63	64	4.0 ml. of pineapple juice	71	83	95	101	114	118	54
7	41	49	62	61	10 mg. of International vitamin B preparation	72	77	84	91	98	102	41

as indicated by a definite decrease in their growth rate, they were arranged in groups of from six to ten animals per group and were fed daily measured quantities of the pineapple juice. The feeding of this juice to groups of rats as a supplement to a vitamin G deficient diet was continued for a period of 8 weeks, during which all observations were accurately recorded.

The results obtained by this series of experiments are presented in table 3.

The data presented above indicate that a daily allotment of 12 ml. of the canned pineapple juice furnished slightly more than 1 Sherman unit of this vitamin. According to this assay, each ounce of this juice contained approximately 2.5 Sherman units of vitamin G.

TABLE 3

Showing the growth stimulating effect of pineapple juice when fed at different levels to groups of rats which were receiving a diet deficient in vitamin G.

DEPLETION PERIOD					SOURCE OF VITAMIN G. QUANTITY FED DAILY	CURATIVE PERIOD								
Group	Average initial weight	Average weight at end of				Average weight at end of								Gain in weight
		7 days	14 days	Depletion period		7 days	14 days	21 days	28 days	35 days	42 days	49 days	56 days	
	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	43	47	53	54	None	56	58	55	53	51	51	49	46	−8
2	42	49	53	53	4.0 ml. of pineapple juice	55	55	56	56	59	59	55	56	3
3	44	53	57	57	6.0 ml. of pineapple juice	63	63	65	64	65	64	63	62	5
4	42	50	52	51	8.0 ml. of pineapple juice	56	54	54	55	55	55	54	55	4
5	44	53	55	56	10.0 ml. of pineapple juice	63	65	64	66	66	68	65	66	10
6	44	51	54	53	12.0 ml. of pineapple juice	62	65	67	68	73	74	76	77	24
7	42	49	53	52	14.0 ml. of pineapple juice	60	64	66	67	69	71	76	81	29

Vitamin C. The vitamin C content of the canned pineapple juice was determined according to the prophylactic technic of Sherman, La Mer and Campbell ('22). Young guinea pigs, ranging in weight from 250 to 300 gm. each, were kept in individual metal cages, which were provided with raised screen bottoms. These animals were arranged in groups of from six to ten individuals per group, and each guinea pig was provided with a weighed but liberal quantity of the scurvy producing diet. Fresh distilled water was provided daily for all animals. Each animal was weighed at weekly

intervals and an accurate record was made of the amount of food consumed. At each weighing period, all residual food remaining from the previous week was discarded and a freshly prepared portion fed.

A supply of the pineapple juice sufficient for 1 week was kept in the refrigerator at all times, and the cans were opened just prior to the feeding of the juice. Starting with the first day of the experimental period, an accurately measured amount of pineapple juice, in a clean earthenware container, was fed daily to each pig. The animals comprising each of the several groups received identical allotments of the juice under test. The duration of the test period ranged from 56 to 90 days. When it became definitely evident that certain groups of pigs were receiving pineapple juice in great excess of that required to protect them against scurvy, such groups of animals were discontinued. Those receiving the higher levels of juice (10 ml. daily) were discontinued after 56 days, while those that received slightly less juice (9 ml. daily) were discontinued at the end of 70 days. All animals that received less than a protective dose of the juice and those that received a protective dose or slightly more than a protective dose were continued on experiment until death ensued or throughout the 90-day period. All animals that died and those that were killed at the termination of the various experiments were autopsied and rated according to the various degrees of scurvy manifested (Sherman, La Mer and Campbell, '22). These various degrees of scurvy (viz., hemorrhage of rib junction, muscles, joints, and intestines; fragility of the jaw bone and of the joints; looseness of the teeth; beading of the rib junctions) were scored from 0 to 3, according to the degree of scurvy manifested by each tissue. According to this method of scoring, a score of 8 indicated definite scurvy, while a score of 24 indicated the highest possible degree of scurvy. The quantity of juice required to produce a score of from 0 to 2 was considered the minimum protective dose.

The data obtained from this series of experiments are presented in table 4. It will be noted that all animals of groups 1 and 2 died during the course of the experiment. It may be of interest to state in this connection that all animals of group 1 were dead by the thirty-first day, while all animals of group 2 were dead by the forty-second day.

From the data presented in table 4, it is evident that canned pineapple juice contains appreciable quantities of vitamin C.

TABLE 4

Showing the results obtained by feeding varying amounts of canned pineapple juice to groups of young guinea pigs that were receiving a scurvy-producing diet

ANIMAL GROUP NO.	NUMBER OF ANIMALS CONSIDERED	QUANTITY OF JUICE FED DAILY	AVERAGE DAILY GAIN IN WEIGHT	AVERAGE SCURVY SCORE
		ml.	*gm.*	
1	6	0	All died	Severe
2	6	3.0	All died	Severe
3	3	4.0	0	17
4	4	5.0	0.5	8
5	9	6.0	1.8	3
6	7	7.0	2.4	0–2
7	5	8.0	2.7	0
8	8	9.0	2.7	0
9	7	10.0	2.9	0

Approximately 7 ml. of this juice were required to furnish a minimum protective dose of this vitamin. From this, it follows that the canned pineapple juice tested contained approximately four minimum protective doses of vitamin C per ounce. Since a minimum protective dose of vitamin C is approximately 10 International units of this vitamin, it would follow from the above scurvy scores that 1 ounce of canned pineapple juice contained approximately 40 International units of vitamin C.

SUMMARY

A biological assay of canned pineapple juice for its vitamin content, using standard biological methods, showed this juice to be a good source of vitamins A and B, a fair source of vitamin C, and to contain a measurable quantity of vitamin G. When expressed in terms of vitamin units, 1 ounce of this juice contained approximately 30 Sherman units of vitamin A, 20 Sherman units of vitamin B, 2.5 Sherman units of vitamin G, and four minimum protective doses or 40 International units of vitamin C.

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THE INFLUENCE OF DEXTRIN AND SUCROSE ON GROWTH AND DERMATITIS

R. C. BENDER, S. ANSBACHER, G. E. FLANIGAN AND G. C. SUPPLEE
The Dry Milk Company Inc., Research Laboratories, Bainbridge, New York

FIVE FIGURES

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In the study of the water-soluble vitamins of the B-complex the carbohydrate constituent of the basal ration appears to be a factor influencing both the incidence of dermatitis and rate of growth. The observations of Hogan and Richardson ('32 and '34), who report a high incidence of dermatitis in rats when using sucrose, and the more recent studies by Guerrant and Dutcher ('34) who observed a marked difference in rate of growth when using different carbohydrates, are particularly significant.

For comparative studies concerning the vitamin B-complex in milk and its derivatives and in rice polish, dextrin, as prepared in these laboratories, has been used as the carbohydrate of the basal rations. This product was made from a refined cornstarch, moistened with a 0.2 per cent citric acid solution, autoclaved for 5 hours at 120°C., and subsequently dried and ground. Various rations containing this dextrin have been used successfully with 2000 or 3000 white rats in comparative studies involving growth rate and polyneuritis. The 'pellagra-like condition' as described by Goldberger and Lillie ('26), the 'characteristic dermatitis' as observed by Hogan and Richardson ('32), the '(a) type of dermatis' as recorded by Chick, et al. ('35), and the 'pellagra-like dermatitis' as described by György ('35) has rarely if ever been noted.

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However, in numerous cases a non-specific skin or fur condition, possibly indicative of an atypical dermatitis, has been observed especially in those animals the period of survival of which has been extended several weeks with test substances known to contain vitamin $B(B_1)$ and possibly small amounts of the other factors of the vitamin B-complex.

The availability of pure vitamin $B(B_1)$ (Merck) and pure lactoflavin as prepared in these laboratories (Supplee, et al., '36) will undoubtedly facilitate study of the vitamin B factors. The fruitfulness of such investigations however, may be predicated upon the carbohydrate employed in the basal ration. The influence of this constituent on the incidence of dermatitis and growth of white rats is shown by the following data wherein pure vitamin B and lactoflavin were used as the primary supplements.

EXPERIMENTAL

As a basis of departure, a basal ration was used containing dextrin, 62 parts; hydrogenated vegetable oil,[1] 10 parts; vitamin-free casein (Labco),[2] 20 parts; salt mixture 40,[3] 4 parts; powdered agar-agar, 2 parts; and cod liver oil, 2 parts. As the primary modification, the dextrin constituent of this ration was entirely replaced by sucrose; as a further modification the hydrogenated vegetable oil content was reduced from 10 to 3 parts and the sucrose content increased to 69 parts.

White rats, 22 to 26 days old and weighing 40 to 50 gm. when placed on the sucrose rations unsupplemented with other dietary factors showed little or no gain and survived for a period of only 2 to 5 weeks. Those on the dextrin rations gained from 2 to 5 gm. per week for a period of 5 to 8 weeks. No evidence of dermatitis has been observed from any of the unsupplemented rations, death occurring before the development of the

[1] Crisco.

[2] The Labco vitamin-free casein is distributed by The Casein Company of America, Inc., New York.

[3] H. Steenbock and E. M. Nelson. 1923. Fat-soluble vitamine. XIII. Light in its relation to ophthalmia and growth. J. Biol. Chem., vol. 56, p. 355.

symptoms. The survival period of the animals receiving the rations containing 10 parts of hydrogenated vegetable oil averaged from 1 to 2 weeks longer than those receiving the unsupplemented rations with the lower fat content.

Obviously a basal ration entirely devoid of the vitamins of the B-complex must be supplemented with one or more of the factors of this complex if a comprehensive experimental plan is to be carried out. To supply the antineuritic factor 2.5 γ or 12.5 γ of the crystalline vitamin B(B_1) (Merck) was fed as a daily supplement. When 12.5 γ supplemented the dextrin

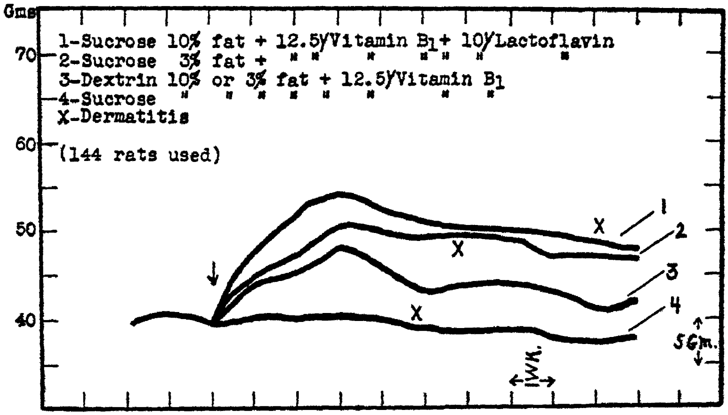

Fig. 1 Growth response of white rats on dextrin and sucrose rations supplemented with crystalline vitamin B_1 and crystalline lactoflavin.

rations some growth resulted for a short period of time, after which the weight remained stationary (fig. 1, curve 3); the survival period was extended over several months. No dermatitis of the character described by the investigators previously mentioned was observed. When the same amount of vitamin B supplement was fed with the sucrose rations containing either 10 or 3 parts of hydrogenated vegetable oil, the growth response was practically nil (fig. 1, curve 4). When 2.5 γ of vitamin B supplemented the sucrose rations the average survival period was shorter than for those animals receiving the same rations supplemented with 12.5 γ.

The significant reaction however, of almost all of the animals receiving the sucrose rations was the development of a dermatitis which is believed to be identical with that described by the various authors previously noted. This manifestation, illustrated in accompanying figure 4, was observed usually as early as the fifth week after starting the vitamin B supplement. The dermatitis also resulted from the sucrose rations with the higher amount of fat, but with less regularity than with the low fat sucrose ration; its appearance with the former was delayed on an average from 3 to 4 weeks.

From these comparisons it would seem that there is a difference in the character of the carbohydrates used, since irrespective of the prolonged survival period of the animals receiving vitamin B and the dextrin ration, no evidence of dermatitis whatsoever was noted. However, when sucrose was employed, irrespective of the level at which vitamin B was fed and irrespective of the fat content of the basal ration, dermatitis developed with marked consistency. As high as 85 per cent of the animals on the sucrose ration containing 3 parts of hydrogenated vegetable oil showed a severe form of dermatitis after 6 to 8 weeks.

In order further to determine whether the sucrose rations would show consistent results in causing dermatitis, they were supplemented with lactoflavin as well as with the pure vitamin B. The response of the animals receiving the low fat sucrose ration supplemented with 12.5 γ vitamin B and 10 γ of pure lactoflavin is shown in figure 1, curve 2, from which it will be noted that the incorporation of 10 γ of lactoflavin caused an immediate, although slight, growth response for a period of 2 to 3 weeks after which the weight remained practically stationary. The dermatitis developed after about 5 to 6 weeks with the same degree of regularity as when the same ration was supplemented with vitamin B only. When the same supplements were fed with the sucrose ration containing the higher amount of fat (fig. 1, curve 1) the incidence of the dermatitis was again delayed for a period of 3 to 4 weeks and its occurrence was not as regular as with the low fat ration.

A further comprehensive comparison of the dextrin and sucrose rations was made involving supplementation with the pure vitamin B, lactoflavin, and a lactoflavin-free material derived from a crude milk vitamin concentrate (Supplee, et al., '31). After an initial depletion period of about 2 weeks all

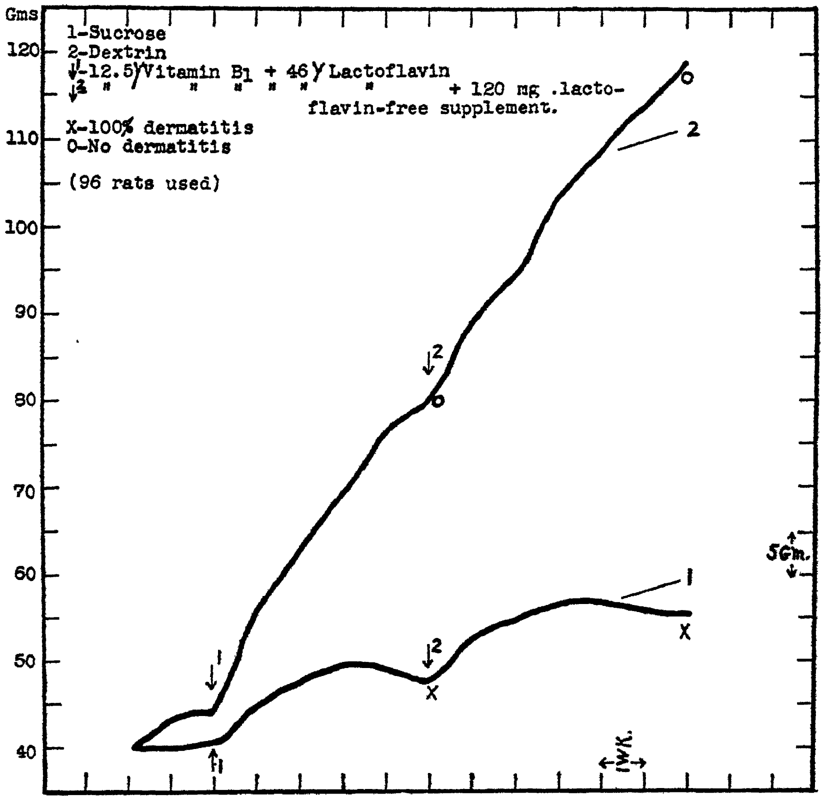

Fig. 2 Influence of dextrin and sucrose on growth and incidence of dermatitis in white rats receiving vitamin B_1 and lactoflavin supplements.

animals on the dextrin and low fat sucrose rations were fed a daily supplement consisting of 12.5 γ of pure crystalline vitamin B and 46 γ of lactoflavin contained in an impure concentrate (2-550) (Supplee, et al., '35). After a 5-week period of feeding these supplements each group was fed the crude milk vitamin concentrate previously freed from lactoflavin,

as an additional supplement in an amount equivalent to 120 mg. of the concentrate.

The animals receiving the sucrose ration (fig. 2, curve 1) supplemented with vitamin B and lactoflavin showed a definite growth response as a result of such supplementation, but declined in weight after about 3 to 4 weeks. At the end of the fifth week 100 per cent of these animals showed the typical dermatitis symptoms. The animals receiving the dextrin ration (fig. 2, curve 2) similarly supplemented, continued to grow at a very satisfactory rate and showed no evidence of

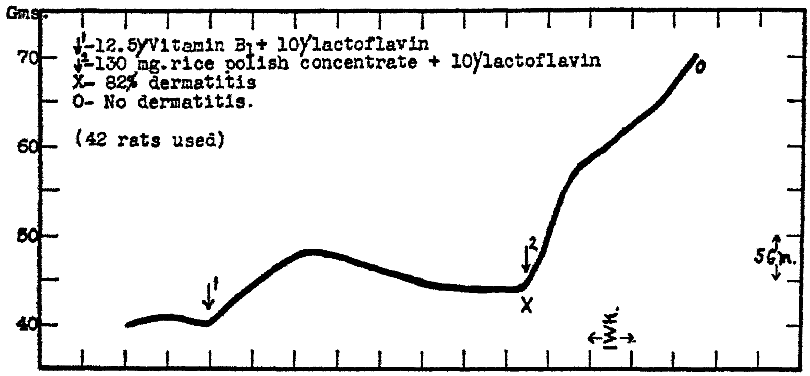

Fig. 3 Influence of vitamin B_1, lactoflavin and rice polish concentrate on growth and incidence of dermatitis in white rats receiving sucrose.

dermatitis. After 5 weeks, when all animals on the sucrose ration showed dermatitis, the lactoflavin-free supplement was added. After feeding this additional supplement for 6 weeks (curve 1) the dermatitis still persisted without apparent diminution in severity. The animals fed this additional supplement with the dextrin ration (curve 2) continued to grow and no evidence of dermatitis whatsoever was noted throughout the entire period of observation. These data seem to indicate that neither the vitamin B nor the lactoflavin, nor the lactoflavin-free material in the form and at the levels at which they were fed, had any influence on the occurrence of the dermatitis.

In order to determine whether the difference in reaction which resulted from the dextrin and sucrose rations may be due to a factor of vitamin character, or a condition in the ceca of the experimental animals (Guerrant, et al., '35) the following experiments were made.

The sucrose ration containing 3 parts of hydrogenated vegetable oil was employed. After the initial depletion period, the animals received vitamin B, lactoflavin and the lactoflavin-free material, all at the same levels as in the previous experiment. These supplements were fed for 7½ weeks with results as shown in figure 3; the curve is the composite average of forty-two individuals. The reaction of the animals both with respect to growth and incidence of dermatitis was substantially the same as previously determined (fig. 2, curve 1). At the 7½-week period 21 per cent of the animals were dead and 82 per cent of the survivors showed the typical dermatitis. At this juncture the supplement of 12.5 γ of pure vitamin B was discontinued and in its place 130 mg. of an especially prepared rice polish concentrate (Supplee, et al., '34) was fed. This amount of the concentrate carried approximately the equivalent of 12.5 γ of vitamin B, and also about 1 mg. of lipid matter and about 30 mg. of non-reducing sugars.

The response of the animals receiving this amount of rice polish concentrate and 10 γ of pure lactoflavin as supplements to the sucrose ration, is shown in the continuation of the curve in figure 3, from which it will be noted that a substantial rate of growth resulted and that the dermatitis began to improve immediately. The accompanying figure 5 illustrates the condition of the animals after about 1 week of feeding of these supplements. Definite signs of healing are clearly evident. After 4 weeks the dermatitis was cured and all the animals appeared to be normal in this respect.

It is evident that these results indicate the presence of an antidermatitis factor in the rice polish concentrate. Supplementary data showed that the small amounts of lipid matter and of non-reducing sugars carried by the concentrate are not the cause of the healing of the dermatitis observed in these

Fig. 4 Characteristic dermatitis resulting from the unsupplemented basal ration containing sucrose.

Fig. 5 Characteristic dermatitis resulting from the unsupplemented basal ration containing sucrose, cured by an aqueous extract of rice polish, 120 mg. daily.

experiments. Furthermore in view of the other experiments reported herein, it may be assumed that dextrin carries some of the antidermatitis factor, or that the favorable reaction of the dextrin fed rats is due to cecal fermentation as indicated by Guerrant, et al. ('35). The data are considered to be conclusive in showing that two of the entities of the vitamin B-complex, namely vitamin B and lactoflavin have no antidermatitis effect whatsoever when employed as supplements to the sucrose rations. Neither do these factors used singly or together permit normal or continued growth unless they are supplemented with a third factor or group of factors.

SUMMARY

1. Comparative data from basal rations containing dextrin and sucrose, respectively, show that no dermatitis resulted when the former was used, whereas a high incidence of dermatitis resulted when sucrose served as the basal carbohydrate.

2. Vitamin B and lactoflavin supplementing the sucrose ration did not prevent the development of dermatitis, nor did these supplements permit normal and continued growth; such supplements fed with dextrin promoted a substantial rate of growth.

3. A concentrate prepared from rice polish cured the dermatitis occurring in the sucrose fed animals and at the same time promoted a substantial rate of growth, provided adequate amounts of vitamin B and lactoflavin were fed simultaneously.

4. The occurrence of dermatitis was delayed and not as regular with a sucrose ration containing 10 per cent hydrogenated vegetable oil as with one containing 3 per cent of the same oil.

5. The data as a whole would seem to indicate that the basal ration containing 69 parts of sucrose and 3 parts of hydrogenated vegetable oil is well suited for the determination of the growth-promoting properties of lactoflavin, provided that it is supplemented with adequate amounts of vitamin B and the vitamin factor or group of factors contained in the rice polish concentrate.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Mr. L. C. Babcock of The Dry Milk Company Research Laboratories' staff.

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LACTOFLAVIN, A NECESSARY GROWTH-PROMOTING DIETARY FACTOR

S. ANSBACHER, G. C. SUPPLEE AND R. C. BENDER
The Dry Milk Company Inc., Research Laboratories, Bainbridge, New York

THREE FIGURES

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The current literature seems to show conclusively that lactoflavin irrespective of its source is a necessary growth-promoting dietary factor. However, the potency of the preparations of different workers varies considerably. Booher ('34) reports that 600 γ per rat per day induced a consistent and continuous growth rate of 5.0 to 6.0 ± 1.0 gm. per week over a 4-week period. Itter, et al. ('35) found that 100 γ produced an average weekly gain of 8 to 10 gm. for 3 weeks. Lepkovsky and collaborators ('35) state that 100 γ per day enabled rats to gain about 26 gm., and 300 γ about 30 gm. in 9 days. Recently Stare ('35) described a crystalline preparation of lactoflavin which gave an average weekly gain in weight of about 4 gm., when 50 γ was fed daily for about 9 weeks. The crystalline products prepared in Karrer's and Kuhn's laboratories are reported to have a considerably higher potency. According to Karrer, et al. ('35) a daily weight increase of 0.75 gm. is obtained, if 3 γ of the less easily soluble fraction of the crystalline lactoflavin is fed daily; v. Euler and co-workers ('34) report that 5 γ per rat per day produces an average daily gain of 1.1 gm. for 5 weeks and that 10 γ induces a daily gain in weight of 1.3 gm. for 5 weeks; Kuhn and his collaborators ('35) indicate that 10 γ of the natural lactoflavin produces a daily gain in weight of 1.45 gm. and 10 γ of the synthetic lactoflavin a gain of 1.43 gm. In view of these data it seems of

interest to report the results which have been obtained with the crystalline lactoflavin prepared in these laboratories (Supplee, et al., '36).

EXPERIMENTAL

The various data and observations recorded in a recent paper from these laboratories (Bender, et al., '36) showed that a sucrose containing basal ration entirely devoid of the vitamins of the B-complex, is well suited for the determination of the growth-promoting properties of lactoflavin, provided that it is supplemented with adequate amounts of vitamin $B(B_1)$ as well as with a third factor or group of factors contained in a specially prepared rice polish concentrate (Supplee, et al., '34). The basal ration consisting of sucrose, 69 parts; vitamin-free casein (Labco),[1] 20 parts; hydrogenated vegetable oil,[2] 3 parts; salt mixture 40,[3] 4 parts; powdered agar-agar, 2 parts; and cod liver oil, 2 parts, was used in the studies reported in the present paper, as this ration has been found to give reliable and consistent results, when fed to white rats, 22 to 26 days of age and weighing 40 to 50 gm.

After the depletion period the animals received various amounts of the rice polish concentrate as a daily supplement. This concentrate contains an insignificant amount, if any, of lactoflavin as determined by the previously described fluorometric method (Supplee, et al., '36) which is capable of detecting as little as 1 part in 20 million. The biological data which are presented in the following paragraphs substantiate the results obtained fluorometrically.

As a further supplement, 12.5 γ of the crystalline vitamin B (Merck) was fed. For determining the growth-promoting properties of lactoflavin, the pure crystalline product (Supplee, et al., '36) was fed simultaneously with either one

[1] The Labco vitamin-free casein is distributed by The Casein Company of America, Inc., New York.

[2] Crisco.

[3] H. Steenbock and E. M. Nelson. 1923. Fat-soluble vitamine. XIII. Light in its relation to ophthalmia and growth. J. Biol. Chem., vol. 56, p. 355.

or both of the above supplements in amounts varying between 2.0 and 20.0 γ.

The results from the feeding of the rice polish concentrate and lactoflavin are illustrated in figure 1, graphs 1 to 3. It is to

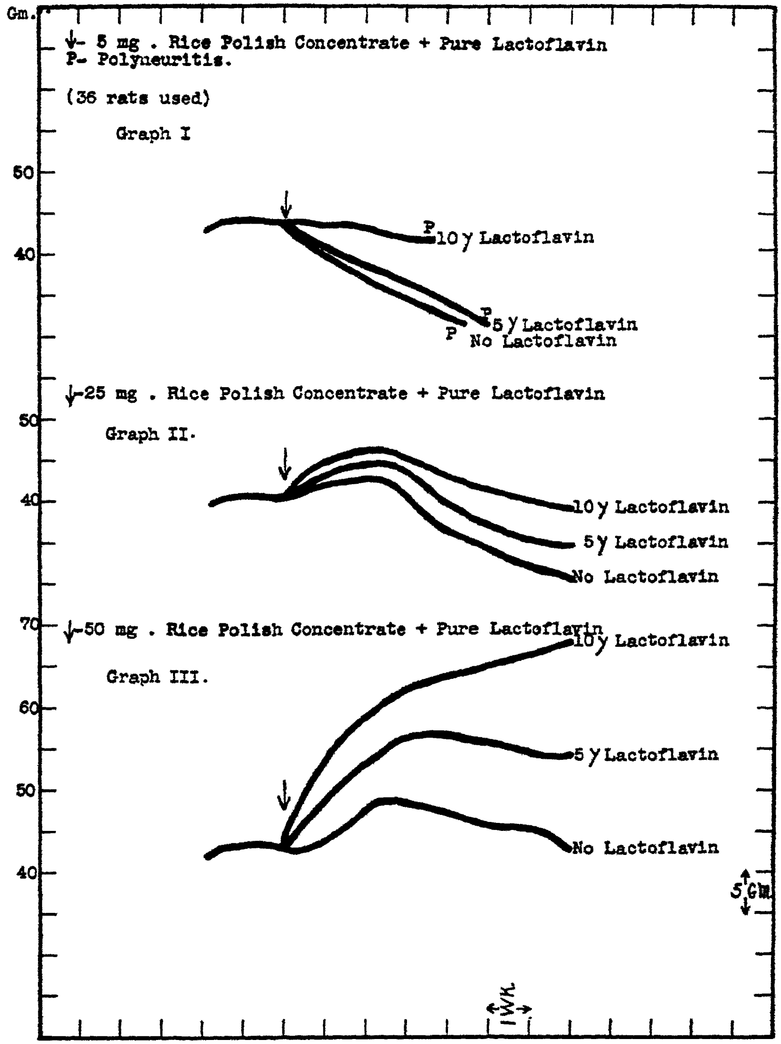

Fig. 1 The effect of pure lactoflavin on the growth of white rats receiving variable amounts of rice polish concentrate.

be noted that a relatively high percentage of the animals receiving the rice polish concentrate at the 5-mg. level (graph 1) developed paralysis and that all died within 4 to 5 weeks from B-avitaminosis, 5 mg. of the concentrate carrying only 0.48 γ of vitamin B as previous assays have shown. It is significant that the weight of the animals receiving the crystalline lactoflavin at the 10.0 γ level remained practically constant throughout the survival period whereas the animals receiving only 5.0 γ or no lactoflavin lost weight rapidly.

The rice polish concentrate fed at the 25-mg. level (graph 2) permitted slight growth for a period of 2 to 3 weeks, followed by a gradual loss in weight. However, the higher the level of lactoflavin the greater was the weight of the animals during the entire period. A few isolated cases of paralysis followed by death occurred indicating that the vitamin B content of 25 mg. of the rice polish concentrate is marginal.

The data obtained from the feeding of the rice polish concentrate at the 50-mg. level (graph 3) show a markedly greater growth than that obtained with the 5- and 25-mg. level and comparable amounts of lactoflavin. No paralysis and no death occurred. The rats receiving no lactoflavin gained only a few grams during the first 2 to 3 weeks and lost weight thereafter. Those receiving 5.0 γ of lactoflavin showed an average gain per week of about 5 gm. during the first 3 weeks and their weight remained practically constant thereafter. The animals receiving 10.0 γ of lactoflavin showed a satisfactory growth rate during the entire experimental period. These data appear to show that lactoflavin is necessary for normal growth.

In order to further substantiate these results and to eliminate the B-avitaminosis noted with the lower levels of the rice polish concentrate, the entire experiment illustrated in figure 1 was repeated, but all the animals received an adequate amount of vitamin B in the form of 12.5 γ of the crystalline product in addition to the rice polish concentrate and lactoflavin supplements (fig. 2, graphs 1 to 3).

When 5 mg. of the rice polish concentrate was fed in conjunction with the vitamin B and varying amounts of lactoflavin (graph 1), most animals survived, but showed the

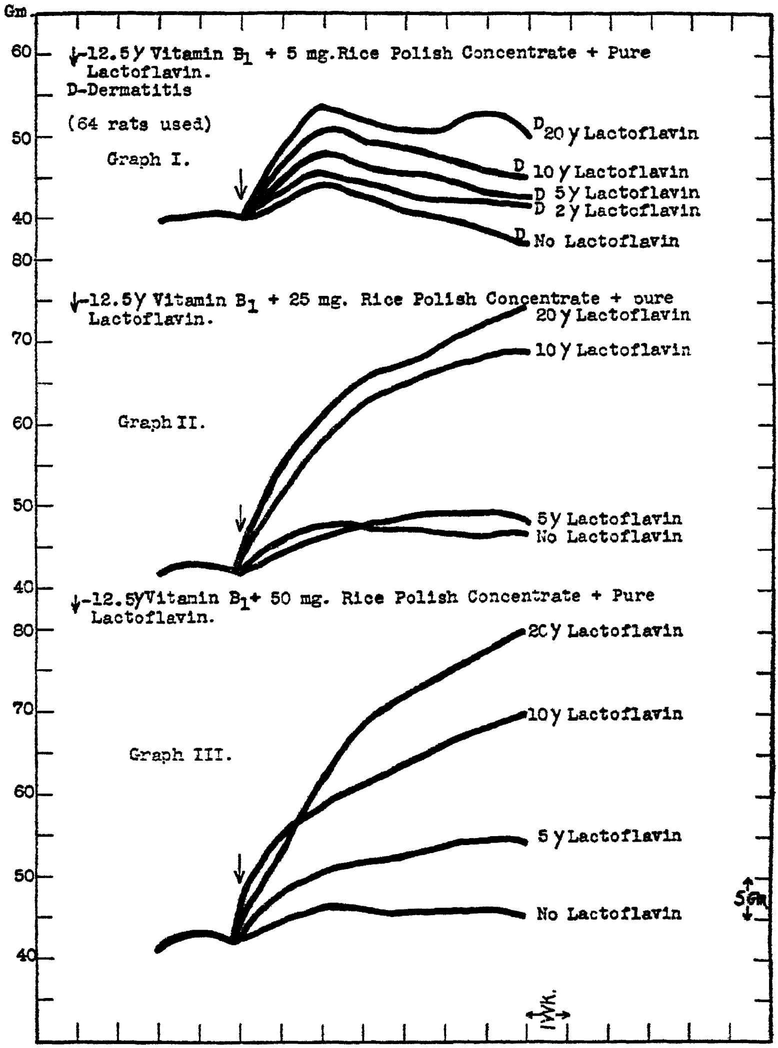

Fig. 2 The effect of pure lactoflavin on the growth of white rats receiving variable amounts of rice polish concentrate and a constant amount of pure vitamin B_1.

typical symptoms of dermatitis indicative of a deficiency in a factor or group of factors other than vitamin B or lactoflavin, as was shown in a previous paper (Bender, et al., '36). The rats fed only 2.0 γ of lactoflavin daily had a higher weight throughout the experimental period than those receiving no lactoflavin. The more lactoflavin fed the greater was the weight of the animals. However, the percentage incidence of dermatitis on the 20.0 γ level of lactoflavin was as high as when no lactoflavin was fed.

When 25 mg. of the rice polish concentrate, 12.5 γ of vitamin B and varying amounts of lactoflavin were fed (graph 2) none of the animals developed dermatitis. However, the weight of the animals receiving 5.0 γ or no lactoflavin remained practically stationary whereas the animals receiving 10.0 and 20.0 γ of lactoflavin, respectively, gained weight at a satisfactory rate throughout the entire experimental period.

The results obtained from the feeding of the 50 mg. of the rice polish concentrate are again clear-cut (graph 3). With 20.0 γ of lactoflavin daily the rats gained 38 gm. in 7 weeks, with 10.0 γ 28 gm., with 5.0 γ 13 gm., and with no lactoflavin the animals gained 4 gm. within the first 2 weeks and their weight remained practically constant thereafter.

The above results were further substantiated by an experiment in which a great number of animals was fed 12.5 γ of vitamin B and varying amounts of lactoflavin for 7½ weeks (fig. 3). At the end of this period the animals were practically constant in weight having shown only a slight growth as a result of the vitamin B and lactoflavin supplementation. During the period the greatest growth was shown by the group receiving 10.0 γ of lactoflavin and a large percentage of all the animals developed dermatitis. After 7½ weeks the vitamin B feeding was discontinued and 130 mg. of the rice polish concentrate was fed daily in its place. The response resulting from this supplement feeding is seen in the continuation of the curves in figure 3. The dermatitis was cured in all the animals. With 10.0 γ of lactoflavin a gain in weight of 36 gm. was obtained in 4 weeks, with 5.0 γ 20 gm., with 2.0 γ 12 gm.,

and with no lactoflavin the animals gained only 5 gm. during the first week, their weight remaining practically constant thereafter. This set of experiments emphasizes again the necessity of lactoflavin for normal growth and confirms that the rate of growth of rats is dependent upon the amount of lactoflavin in their feed.

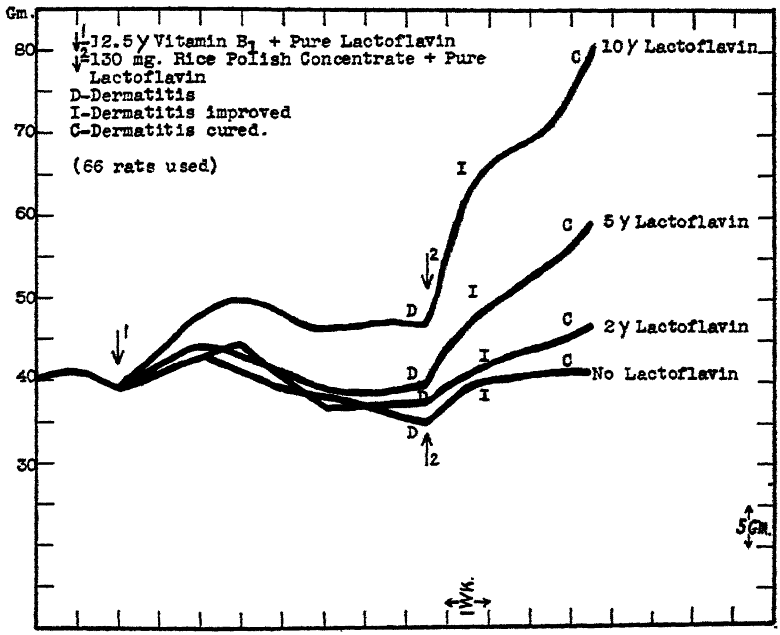

Fig. 3 The influence of vitamin B_1, rice polish concentrate and variable amounts of pure lactoflavin on growth and incidence of dermatitis in white rats.

DISCUSSION

The data as a whole seem to show conclusively that lactoflavin is a necessary growth-promoting dietary factor. The various results obtained point to the complexity of the biological assays for the various vitamins of the B-group. The vitamin B appears to be a prerequisite of such assays; a supplement feeding of 12.5 γ of the pure crystalline product which according to the manufacturer's data corresponds to 5 units of vitamin B, provides adequate amounts of the antineuritic

factor. The factor or group of factors which prevents and cures dermatitis and which is carried by the rice polish concentrate employed must likewise be supplied in adequate amounts, if the true growth-promoting properties of lactoflavin are to be determined.

Under such conditions the lactoflavin prepared in these laboratories compares well in its growth-promoting properties with the most active products prepared by other investigators. As little as 2.0 γ of lactoflavin daily was found to be sufficient to influence the rate of growth of white rats.

As to the potency of lactoflavin when expressed in terms of units, the above data would seem to permit the conclusion that 100 gm. of the pure crystalline lactoflavin corresponds to about 15 million units. This calculated potency is identical with that which Scheer ('35) has recently indicated. However, it should be emphasized that there is apparently a discrepancy between the potency of the pure lactoflavin and that contained in natural products, as v. Euler et al. ('34) have already pointed out. It seems therefore that expressing the potency of lactoflavin in terms of units might lead to similar controversies as those which are continuously appearing in the literature concerning the potency of the pure vitamin D and that contained in such biological substances as e.g., irradiated milk. If one considers furthermore that a pure vitamin or enzyme corresponds to less units than when the same amount of the vitamin or enzyme is a prosthetic group (Ansbacher and Supplee, '34) in a symplex (Willstätter and Rohdewald, '34), it becomes very doubtful whether the potency of biological substances such as vitamins and enzymes, can be expressed in units as determined by empirical procedures.

SUMMARY

Lactoflavin is a necessary growth-promoting dietary factor. A difference in the rate of growth of white rats results from the differences in the daily intake level of pure crystalline lactoflavin varying from 2.0 to 20.0 γ.

The growth-promoting properties of lactoflavin are readily determined with a suitable basal ration adequately supplemented with pure vitamin B and a third factor or group of factors necessary for the prevention and cure of dermatitis and carried by rice polish.

The potency of lactoflavin may be 'calculated' to be 150,000 units per gram. However, as briefly discussed, the 'unit' designation for expressing the potency of biological substances, such as vitamins and enzymes, may be meaningless or erroneous.

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LESIONS OF THE NERVOUS SYSTEM IN VITAMIN DEFICIENCY

IV. THE EFFECT OF CAROTENE IN THE TREATMENT OF THE NERVOUS DISORDER IN RATS FED A DIET LOW IN VITAMIN A

H. M. ZIMMERMAN AND GEORGE R. COWGILL

Departments of Pathology and Physiological Chemistry, Yale University School of Medicine, New Haven, Connecticut[1]

TWO FIGURES

(Received for publication January 16, 1936)

In a previous communication (Zimmerman, '33) which detailed the lesions in the nervous systems of albino rats fed a diet low in vitamin A, it was shown that the animals developed muscular weakness and incoordination after a prolonged subsistence on the diet and after other signs of the deficiency, such as loss of weight and xerophthalamia, supervened. It was further shown that the neurologic manifestations were associated with degeneration of the medullary sheaths of the peripheral nerves and of the spinal cord. In every instance the animals displaying these nervous disorders were necropsied shortly after the appearance of the latter and it seemed worthwhile, therefore, to study in a new experiment the anatomic lesions at various periods in the recovery from these nervous manifestations. With the availability of carotene as a source of vitamin A, it was felt that this ideal could be realized. The following experiments were in part devised to test the effectiveness of carotene in preventing, alleviating or curing these manifestations. Moreover, it was hoped that

[1] The expenses of the study were defrayed in part by a grant from the Research Fund of the Yale University School of Medicine.

if carotene alone proved effective, an answer would be at hand to the question of the role which an absence of unsaturated fatty acids in the diet plays in the production of these nervous lesions.

Shortly after the appearance of the previous paper, some doubt was cast by Grinker and Kandel ('33) on the relation of vitamin A-deficiency to changes in the nervous system. The present communication also deals in part, therefore, with experiments devised to test the reproducibility of the results previously reported.

EXPERIMENTAL PROCEDURE

A total of forty-three albino rats, the offspring of carefully selected and dieted animals maintained in the laboratory of physiological chemistry for breeding purposes, was employed in this study. These animals received the artificial ration low in vitamin A after weaning, which occurred between 21 and 24 days of age. The rats employed as controls received daily, in addition to this ration, 2 drops of a 0.1 per cent solution of carotene in cottonseed oil; one drop of this solution was equivalent to 25 micrograms (25 γ). A similar amount of carotene and sometimes, as will be specified below, double this amount was employed in the 'treatment' experiments.

Diet employed. The composition of the artificial ration low in vitamin A was:

	per cent
Casein (vitamin-free)[2]	18
Corn starch	57.8
Hydrogenated vegetable oil[3]	20
Osborne-Mendel salt mixture IV	4
Linoleic acid	0.2

Linoleic acid was added to the ration in order to supply an adequate amount of unsaturated fatty acid. The hydrogenated vegetable oil used is known to contain such acids, but since their amount may vary from time to time it seemed advisable to incorporate in the ration an adequate supply in the form of linoleic acid.

[2] From the Harris Laboratories, Tuckahoe, New York.

[3] Crisco.

The vitamin B complex was supplied to all rats daily in the form of 0.3 gm. of dried yeast.[4] Vitamin D was supplied in the form of a solution of irradiated ergosterol in cottonseed oil, 17 International Units being supplied daily. Vitamin E is present in sufficient quantity in the vegetable oil; vitamin C is apparently not needed by the rat.

Grouping of animals. The rats were grouped into five classes in accordance with experimental conditions specified for each group.

Group I was composed of nine animals which were maintained on the artificial, vitamin A-low diet from weaning until the end of the experiment. At no time did these animals receive any carotene or other source of vitamin A. The purpose of this experiment was simply to determine whether the results described in the previous communication (Zimmerman, '33) were reproducible.

Group II comprised a total of fifteen animals which were maintained on the same artificial ration low in vitamin A, but which were treated with carotene when marked loss of weight, xerophthalmia and often also 'paralysis' supervened. On the whole, these animals were ineffectively, and probably also inadequately, treated as will be brought out below. In each instance death ensued spontaneously, with but one exception, within 1 to 5 days after the beginning and in spite of therapy. This group in reality represents an 'accident' in this study, for at the beginning of the experiment the intention was to treat the animals adequately. Either because therapy was instituted too late in the course or because insufficient dosage was employed, the animals failed to survive.

Group III consisted of six rats which were effectively treated with carotene after developing loss of weight, xerophthalmia and frequently also 'paralysis,' when fed the artificial diet low in vitamin A. This group differs from group II in that each of these animals began to increase in weight, had a restitution to normal of its ophthalmic condition and in some instances showed an amelioration of its neurologic condition

[4] From the Northwestern Yeast Company, Chicago, Illinois.

as judged by its behavior. In each instance the animals belonging to this group were killed with ether after a prolonged period of recovery sufficient to indicate the effectiveness of carotene as a therapeutic agent in this condition.

Group IV was composed of seven animals fed the artificial ration and 2 drops of a 0.1 per cent solution of carotene in cottonseed oil from weaning until the termination of the study. These animals constituted the positive controls. One animal from each litter was placed in this group; the remainder of the litter mates were scattered among the other groups.

Group V was composed of six rats which were originally started on the vitamin A-low diet but were soon, at various times for each animal, given the basal dosage (2 drops daily of carotene) and were thereby converted into control animals. The reason for this change differed for each animal, but was usually due to the fact that the animal ate poorly and lost weight too rapidly or refused to eat the allotted yeast and thereby may have developed a complicated deficiency or showed other complicating features, such as, unusual weakness and early infection of the urinary tract.

Neurological manifestations. During the progress of these experiments the animals were examined twice weekly until the first sign of a change in their condition, when examinations were made daily or on alternate days. At such times accurate record was made of the body weight, amount of food and vitamin consumption and general physical condition. Special attention was paid to evidence of xerophthalmia and neurologic manifestations. A few simple tests were employed to help in the study of the nervous changes, and these were as follows:

1. Observing the gait on a smooth, level surface, noting especially any tendency to drag the posterior extremities.

2. Holding the animal in a vertical position by the skin of the neck and back to note the posture of the posterior extremities. Paralyzed or weak extremities are hyperextended whereas normal extremities are flexed. This was previously described by Aberle ('34).

3. Climbing over obstacles.
4. Maintaining position on a rotating, loose-meshed wire screen. Records of the performance in these tests were made with the cinema for the comparison of each animal's ability at different times and also with that of the control animals. Occasionally, in spite of these tests, it was difficult to determine whether the neurologic manifestations were simply weakness, muscular incoordination, paresis or actual paralysis. For the sake of brevity in the following tables, all of these neurologic changes were designated by the term 'paralysis,' even if this term was not strictly descriptive of the true condition in a particular instance. The degree of severity of 'paralysis' was designated as moderate (+) or marked (++); weakness was usually designated by the symbol $\pm$.

Material studied and technic employed. Both sciatic nerves, the brachial plexuses, the entire spinal cord and the cerebrum of each animal were removed at necropsy and fixed at once in the proper fixatives. The postmortem examinations were performed immediately after the animals were killed with ether; when the animals died spontaneously the examinations were often unavoidably delayed for 1 hour, but in no instance for more than 2 hours.

The fixatives employed and the methods of fixation of the tissues were the same as those described in the previous communication (Zimmerman, '33). The tissues were sectioned as before and were stained by the Nissl, Marchi, Spielmeyer and Kulschitzky methods and with Sudan III.

RESULTS

Macroscopic examination of the brains, spinal cords and peripheral nerves of all the animals failed to reveal any abnormalities.

In none of the animals were any lesions demonstrable in the cerebrum by any of the staining methods. In every instance was the degree of myelin degeneration in the sciatic nerves more marked than in the brachial plexuses.

Group I. Nine rats fed diet low in vitamin A from beginning until termination of experiment. The essential data as concerns the duration of subsistence of each animal on the diet, the weight curve, the clinical manifestations of vitamin A-deficiency and the extent of the anatomic lesions produced thereby, are summarized in table 1.

All the animals in this group except rats 37 and 45 developed xerophthalmia as evidence of their vitamin A-deficiency. Two (rats 2 and 16) showed clinical signs of 'paralysis' of moderate degree, and two other animals (18 and 24) showed signs of muscular weakness. In the former two there was moderate

TABLE 1

Nine rats fed diet low in vitamin A

ANIMAL NUMBER	DURATION IN DAYS OF EXPERIMENT	WEIGHT IN GRAMS			XEROPHTHALMIA	'PARALYSIS'	DEATH	LESIONS	
		Initial	Maximum	Terminal				Peripheral nerves	Spinal Cord
2	59	70	138	98	+	+	Spontaneous	+	++
8	78	60	150	92	+	—	Spontaneous	+	+
9	64	58	120	106	+	—	Spontaneous	±	±
16	50	50	96	72	+	+	Spontaneous	+	++
17	59	58	106	98	+	—	Spontaneous	+	+
18	55	60	119	98	+	±	Spontaneous	+	+
24	94	53	192	116	+	±	Ether	+	+
37	43	45	98	72	—	—	Spontaneous	—	—
45	62	40	120	120	—	—	Ether	±	±

demyelination of the peripheral nerves and marked degeneration of the medullary sheaths in all the tracts of the spinal cord (fig. 1). In the latter two animals, both the peripheral nerves and the spinal cords showed a moderate degree of degeneration of the medullary sheaths. Again, two animals (8 and 17) which did not show any clinical evidence of neuromuscular disturbance showed moderate demyelination of the peripheral nerves and spinal cords. Rats 9 and 45 which had no clinical signs of nervous disease revealed equivocal changes (±) in their nervous systems. Animal 37, which probably had no vitamin deficiency although subsisting on the deficient ration for 43 days, showed neither clinical nor anatomic evi-

dence of nervous disease; this might be taken as suggesting that a period of 43 days under the conditions of these experiments is not long enough for the production of obvious specific signs of vitamin A-deficiency.

Group II. Fifteen rats fed diet low in vitamin A. Inadequate treatment with carotene. The animals in this group,

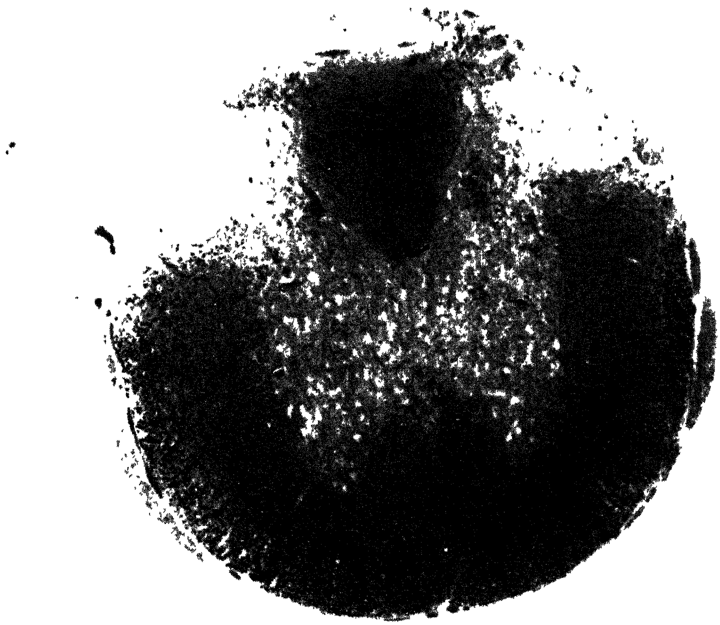

Fig. 1 Rat 16. Thoracic portion of spinal cord showing marked degeneration (black granules) of medullary sheaths in sensory and motor tracts. Marchi stain. × 12.

whose pertinent data are summarized in table 2, all developed xerophthalmia. Five of them (rats 3, 15, 23, 47 and 49) also showed definite signs of 'paralysis' and a sixth one (rat 27) had neuromuscular weakness. The animals without neurologic manifestations all showed a definite loss of weight which in some instances was alarming. In every instance the severity of the symptoms was the deciding factor in the institution of treatment with carotene, which was given in daily

dosage of 2 drops of a 0.1 per cent solution in cottonseed oil. The ineffectiveness of this therapy can be seen from the fact that all but one animal (rat 23) died spontaneously in from 1 to 5 days.

All the animals showed some degree of anatomic change in their nervous systems, which was marked (++) in three of them. The lesions, as in group I, consisted of demyelination of the sciatic nerves, brachial plexuses and spinal cords.

Fig. 2 Rat 51. Lower thoracic portion of spinal cord showing severe grade of degeneration of medullary sheaths (gray zone on periphery). Kulschitzky stain. × 10.

There was no evidence of a preferential or selective involvement of any one of the spinal fiber tracts.

Group III. Six rats fed diet low in vitamin A. Adequate treatment with carotene. Shortly after these animals developed clinical signs of vitamin A-deficiency as characterized by xerophthalmia, and before marked loss of weight ensued, they were started on carotene. The animals which showed signs of weakness or paralysis were given twice the daily dosage (4 drops of a 0.1 per cent solution or 100 micrograms). The animals which failed to show rapid signs of improvement

with the usual dosage of 2 drops were also given the larger amount, and thus all the animals in this group received 4 drops of carotene daily. Reference to table 3 reveals the fact that none of the animals died spontaneously. In every instance the course was terminated after the xerophthalmia was cured and the animal had made a remarkable gain in weight. In every case, also, the animal could obviously have

TABLE 2

Inadequate treatment of vitamin A-deficiency with carotene

Animal number	Duration in days of experiment	Weight in grams			Xerophthalmia	'Paralysis'	Treatment (days)	Death	Lesions	
		Initial	Maximum	Terminal					Peripheral nerves	Spinal cord
3	60	68	134	86	+	+	1	Spontaneous	—	+
5	54	70	142	122	+	—	1	Spontaneous	+	+
7	61	60	152	114	+	—	2	Spontaneous	+	±
11	61	64	134	88	+	—	2	Spontaneous	+	±
12	72	62	131	86	+	—	2	Spontaneous	+	++
14	60	58	112	86	+	—	1	Spontaneous	+	+
15	52	52	92	70	+	+	1	Spontaneous	±	+
23	82	58	198	116	+	+	1	Ether	±	+
27	84	58	212	134	+	±	1	Spontaneous	++	++
31	52	51	96	62	+	—	1	Spontaneous	—	+
32	45	52	101	94	+	—	1	Spontaneous	±	+
41	47	46	98	91	+	—	3	Spontaneous	+	+
42	51	40	94	80	+	—	3	Spontaneous	±	+
47	40	33	88	88	+	+	5	Spontaneous	++	++
49	40	32	92	84	+	+	1	Spontaneous	+	+

lived on indefinitely as far as its previous vitamin A-deficiency was concerned, were the experiment not arbitrarily terminated.

In table 3 the 'maximum weight' is that which the animal achieved while still being fed the deficient ration. It will be noted that animals 48, 50, 51 and 52 made remarkable gains which are listed under 'terminal weight.' Even animals 10 and 29, which were not given carotene over as long a period of time as the others, were well on the up-grade in their weight curve at the termination of the experiment.

In spite of these signs of improvement, the neurologic manifestations remained unimproved throughout the course. Indeed, the clearest picture of the paralysis was obtained after the xerophthalmia had cleared and the animals began to gain weight. The severest grade of degeneration of the nervous system in any of the experimental groups was noted anatomically also in these animals. Degeneration of the medullary sheaths was seen with equal clarity in the Marchi, Sudan III, Spielmeyer and Kulschitzky preparations (fig. 2).

Group IV. Seven control animals These rats, which received the standard artificial ration deficient in vitamin A and

TABLE 3

Adequate treatment of vitamin A-deficiency with carotene

ANIMAL NUMBER	DURATION IN DAYS OF EXPERIMENT	WEIGHT IN GRAMS			XEROPH-THALMIA	'PARALYSIS'	TREATMENT (DAYS)	DEATH	LESIONS	
		Initial	Maximum	Terminal					Peripheral nerves	Spinal cord
10	75	64	150	92	+	—	12	Ether	++	++
29	49	51	110	104	+	—	4	Ether	+	++
48	95	30	82	194	+	+	30	Ether	++	++
50	95	28	88	206	+	+	29	Ether	++	++
51	95	35	104	246	+	+	29	Ether	++	++
52	95	33	97	182	+	+	31	Ether	++	++

supplemented with 2 drops of carotene daily, subsisted on this regimen from 95 to 178 days. They gained in weight steadily from the beginning to the termination of the experiment. None of them developed xerophthalmia and none showed any clinical evidence of neurologic change. Microscopically their nervous systems were entirely normal.

Group V. Six rats converted into control animals late in experiment. For reasons already specified under 'experimental procedure,' these animals were converted into controls with a daily dosage of two drops of carotene after they had received the vitamin A-low diet for various periods of time. Each was killed with ether at the termination of the experiment which lasted from 101 to 170 days. One animal

(rat 26) was started on carotene before any signs of vitamin A-deficiency, including xerophthalmia, had developed. At the first signs of ocular abnormality, the remaining five animals (rats 25, 30, 40, 43 and 44) were started on a daily course of carotene administration. By that time, however, rat 40 had shown some weakness of its posterior extremities. Only two of the animals, rats 26 and 44, showed very slight anatomic changes of demyelination in their peripheral nerves and spinal cords. These changes were, indeed, so slight as to be of questionable significance. The remaining four animals had intact nervous systems on microscopic examination.

COMMENT

The results of the experiment with the animals in group I confirm the findings reported in the previous paper (Zimmerman, '33); namely, that rats subsisting on an artificial ration low in vitamin A often develop neurologic manifestations of disease which are due to demyelination of the peripheral nerves and spinal cord. These manifestations develop some time after the appearance of xerophthalmia, which is characteristic of vitamin A-deficiency, and in general there is a parallel between their severity and the degree of anatomic change in the nervous system. There is a certain amount of individual variation in these animals as regards the time of onset of the symptoms and the survival period. When untreated, however, these animals invariably succumb to the deficiency. Even those rats which succumb prior to the onset of any nervous symptoms show some degree of anatomic change in their nerves and spinal cords. It is quite conceivable that the clinical expression of these lesions is prevented in such instances by the intervening death of the animals.

What has just been stated concerning the results with the animals in group I applies equally to the results obtained with the animals in group II.

The experience with carotene as a therapeutic measure for the vitamin A-deficiency produced in this group of animals seemed disappointing. Its only effect was a possible prolonga-

tion of life for a day or two, but even this is questionable. Since it was realized that the animals were being treated late in their course, it was resolved to institute therapy earlier and to employ larger dosages in the experiment with group III. Here the effectiveness of carotene was shown beyond reasonable doubt. All the animals made remarkable improvement in their condition except for their neurologic symptoms.

The lesions in the nervous system were most severe in this group of animals, but this after all is not surprising in view of the fact that the nervous system has such poor ability to regenerate. The injury produced in it during the regime on the vitamin A-low diet is a permanent one and possibly even progressive. Thus the hope to be able to treat effectively the nervous disorder with carotene did not materialize, but an opportunity was provided to study its anatomic counterpart at various periods in the recovery from this vitamin deficiency.

In the experiment with group IV it was shown that carotene, as the only source of vitamin A, is effective in preventing all the symptoms and signs of this vitamin deficiency. Furthermore, the results of the experiment with group V indicate that carotene is equally effective in the prevention of nervous disorders in animals which have been deprived of this vitamin for various short periods of time.

Finally, these experiments as a whole show conclusively that the lesions in the nervous systems of animals subsisting on this artificial ration low in vitamin A are not dependent on an absence of unsaturated fatty acids. The presence of linoleic acid in the diet does not prevent these lesions from occurring, whereas the administration of carotene does accomplish this end.

CONCLUSIONS

1. Albino rats subsisting on an artificial ration adequate in all dietary essentials as far as is known except vitamin A develop a nervous disorder which is characterized by muscular weakness, incoordination and in the severest cases, paralysis of the posterior extremities.

2. The anatomic counterpart of this disorder is a demyelination of the peripheral nerves, most extensive in the sciatics, and of scattered fiber tracts in the spinal cord.

3. Carotene, when administered late in the course of this dietary deficiency or in inadequate (50 micrograms) dosage, does not alter the fatal outcome in any appreciable manner.

4. When administered relatively early in the course of the deficiency, and especially in adequate (100 micrograms) dosage, carotene is effective in restoring these animals to good health, the neurologic disorder alone persisting.

5. Animals thus treated with carotene resume their normal rate of growth and recover from the xerophthalmia, but their anatomic changes in the nervous system are even more marked than in the untreated animals. This is attributed to the relative inability of the nervous system to regenerate when once severely injured.

6. All evidence of this dietary deficiency is lacking in animals which receive the same artificial ration when the latter is supplemented with carotene (50 micrograms daily) throughout the experiment.

7. This study indicates that the changes in the nervous systems of animals fed the artificial diet low in vitamin A are due to a deficiency of this factor and not of unsaturated fatty acids.

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THE EFFECTS OF BREED CHARACTERISTICS AND STAGES OF LACTATION ON THE VITAMIN C (ASCORBIC ACID) CONTENT OF COW'S MILK [1]

RUSSEL RASMUSSEN AND N. B. GUERRANT
Department of Agricultural and Biological Chemistry

AND

A. O. SHAW, R. C. WELCH AND S. I. BECHDEL
Department of Dairy Husbandry, Pennsylvania State College

ONE FIGURE

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While milk is not usually considered a vitamin C rich food, from the standpoint of infant feeding it does constitute an important source of this dietary essential. In fact Hess ('20) has reported that one pint of fresh raw milk per day furnishes sufficient vitamin C for the average infant. Chick, Hume and Skelton ('20) found, however, that about 85 ml. of milk were required to furnish a minimum protective dose of vitamin C for the guinea pig. Hart, Steenbock and Ellis ('20) have reported that 50 ml. of summer milk or 75 ml. of winter milk were required to furnish a minimum protective dose of vitamin C, while Dutcher, Eckles, Dahle, Mead and Schaefer ('20) reported that the quantity of milk required to furnish this amount of vitamin C depends upon the diet of the cow and was found to range from 20 to 60 ml.

Results of investigations other than those reported above emphasized the importance of the diet of the cow on the vitamin C content of the milk produced, but a review of the available literature failed to indicate that any systematic

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study had been made concerning the effect of breed characteristics or of stage of lactation on the vitamin C content of milks produced under comparable conditions.

Since milk constitutes an important item in the human diet and since the importance of an adequate amount of vitamin C in the diet is becoming more fully appreciated, it appeared to us that any investigation that might yield further information as to how milks containing greater quantities of this dietary essential might be produced would be worth while.

The more recent investigations concerning the nature and the reactions of vitamin C (ascorbic acid) (King and Waugh, '32; Waugh and King, '32; Harris and Ray, '33; Herbert, Hirst, Percival, Reynolds and Smith, '33; Reichstein, Grussner and Oppenauer, '33; Ault, Baird, Carrington, Haworth, Herbert, Hirst, Percival, Smith and Stacey '33; Bessey and King, '33) appeared to suggest a more direct technic that might be used in carrying out such an investigation. This, together with the fact that the college herd is composed of cows of the various standard breeds, representing different stages of lactation and receiving a uniform diet of definite composition, offered further inducement toward such a study. In fact many of the essentials for a comprehensive study of the effects of both breed characteristics and stages of lactation on the vitamin C content of cow's milk were available.

EXPERIMENTAL

Samples of freshly drawn milk (morning) from cows comprising the college herd were collected and taken directly to the laboratory where the determination of their ascorbic acid content was started at once. These cows were receiving a typical dairy ration which contained weighed quantities of green feed. The ascorbic acid content of the various samples of milk was determined in the following manner: 25 ml. of the fresh milk were treated with 25 ml. of a 16 per cent trichloracetic acid solution in order to precipitate the proteins. The supernatant liquid was removed from the precipitated materials by centrifuging and decanting. The precipitated residue was then washed successively with 20 and 10 ml.

portions of an 8 per cent trichloracetic acid solution. These washings were combined with the supernatant liquid and the total volume titrated with a standardized solution of 2–6 dichlorophenolindophenol. This 2–6 dichlorophenolindophenol was prepared by dissolving 0.1 gm. of the dye in successive portions of warm distilled water, filtering and diluting to 200 ml. The filtered dye solution was then standardized against a N/100 iodine solution and also against pure ascorbic acid before being used. A fresh dye solution was prepared for each day's titration.

DATA

Since space does not permit the presentation of data obtained from each individual cow used in the studies pertaining to the effects of breed characteristics on the ascorbic acid content of the milk, only the data from cows selected as representative of each of the five respective breeds are presented in table 1. In selecting these representative cows, due consideration was given to such factors as stage of lactation, age of cow, milk production, etc. In addition, all cows which showed any detectible evidence of having mastitis were eliminated from consideration. When considered, therefore, from the standpoint of these factors, the cows comprising the several groups were as nearly representative of the respective breeds as was possible to select from the number comprising the college herd.

In the studies relative to the effect of the stage of lactation on the ascorbic acid content of milk, data from all cows were considered in the tabulation. That, is, all cows regardless of breed, age, milk production, etc., were tabulated in monthly groups in accordance with the stages of lactation. The average ascorbic acid contents of the milks for the various stages of lactation were then calculated in terms of milligrams per quart and are presented graphically in figure 1.

DISCUSSION

In the studies relative to the influence of breed characteristics on the ascorbic acid content of cow's milk, the milks

TABLE 1

Giving the ascorbic acid content of a series of milk samples produced by representative cows from five dairy breeds

BREED	NUMBER OF COW	APPROXIMATE AGE OF COW AT START OF EXPERIMENT	ASCORBIC ACID CONTENT OF MILK PER QUART						
			Sample taken 8/1/34	Sample taken 8/4/34	Sample taken 8/11/34	Sample taken 8/20/34	Sample taken 9/1/34	Sample taken 9/8/34	Average
		months	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Ayrshire	1392	92	10.4	12.1	10.5	11.1	12.6	12.2	11.5
Ayrshire	1439	81	14.7	14.6	13.9	17.0	14.2	15.3	14.9
Ayrshire	1477	77	14.3	10.6	12.2	12.5	12.7	12.0	12.4
Ayrshire	1754	39	14.9	12.6	10.6	14.8	14.7	12.2	13.3
Ayrshire	1758	39	8.4	10.0	10.8	13.3	13.3	12.2	11.3
Ayrshire	1770	37	15.9	12.9	13.9	14.2	13.9	12.5	13.9
Average for the breed		61	13.1	12.1	12.0	13.8	13.6	12.7	12.9
Brown Swiss	1551	72	14.6	15.2	17.7	15.2	15.3	12.2	15.0
Brown Swiss	1559	70	11.6	11.6	9.7	10.5	16.3	13.8	12.2
Brown Swiss	1611	64	13.3	14.8	12.2	12.5	13.5	13.4	13.3
Brown Swiss	1615	64	17.6	19.8	19.2	22.0	20.0	18.3	19.5
Brown Swiss	1619	67	12.5	11.5	10.5	12.0	14.6	12.0	12.2
Brown Swiss	1734	42	15.9	15.0	15.7	16.9	16.8	18.0	16.4
Average for the breed		63	14.3	14.6	14.2	14.9	16.1	14.6	14.8
Guernsey	1484	118	12.7	12.6	14.6	16.0	17.1	14.2	14.5
Guernsey	1545	79	12.6	11.5	11.1	13.1	14.7	12.4	12.6
Guernsey	1548	76	11.9	10.5	11.8	13.1	13.5	11.7	12.1
Guernsey	1767	40	14.6	14.9	11.2	11.4	12.5	12.4	12.8
Guernsey	1771	37	18.5	14.3	14.3	16.5	16.3	17.1	16.2
Guernsey	1797	34	12.5	11.4	8.9	7.0	9.9	8.4	9.7
Average for the breed		64	13.8	12.5	12.0	12.8	14.0	12.7	13.0
Holstein	1440	83	9.4	10.7	9.4	9.8	9.8	10.0	9.9
Holstein	1646	58	9.8	10.1	10.0	11.2	11.3	11.7	10.7
Holstein	1656	56	9.9	8.3	10.1	8.5	10.3	10.0	9.5
Holstein	1698	52	11.7	9.5	8.6	8.6	11.3	12.7	10.4
Holstein	1744	41	11.0	10.2	11.3	12.5	10.5	10.1	10.9
Holstein	1805	32	9.2	7.0	7.3	10.8	8.4	7.8	8.4
Average for the breed		54	10.2	9.3	9.4	10.2	10.3	10.4	10.0
Jersey	1480	74	15.2	15.4	14.2	15.3	15.2	15.9	15.2
Jersey	1501	77	10.1	11.4	10.4	12.9	12.8	10.1	11.3
Jersey	1703	112	15.4	14.2	11.3	14.6	13.5	13.9	13.8
Jersey	1717	69	15.8	14.6	14.6	15.1	17.1	19.3	16.1
Jersey	1793	53	13.1	12.5	10.0	8.4	14.1	12.1	11.7
Jersey	1821	46	12.1	13.5	12.4	7.4	12.1	11.8	11.6
Average for the breed		72	13.6	13.6	12.2	12.3	14.1	13.8	13.3

from a total of seventy-nine cows were collected. This group of cows was composed of: 14 Ayrshires, 13 Brown Swiss, 9 Guernseys, 27 Holsteins and 16 Jerseys. A few of these cows were known to have mastitis, but when it was found that mastitic milks gave abnormal ascorbic acid values such cows were eliminated from consideration. Milks from sixty-one cows were titrated at each of the six titration periods, while milks from eleven other cows were titrated from two to five

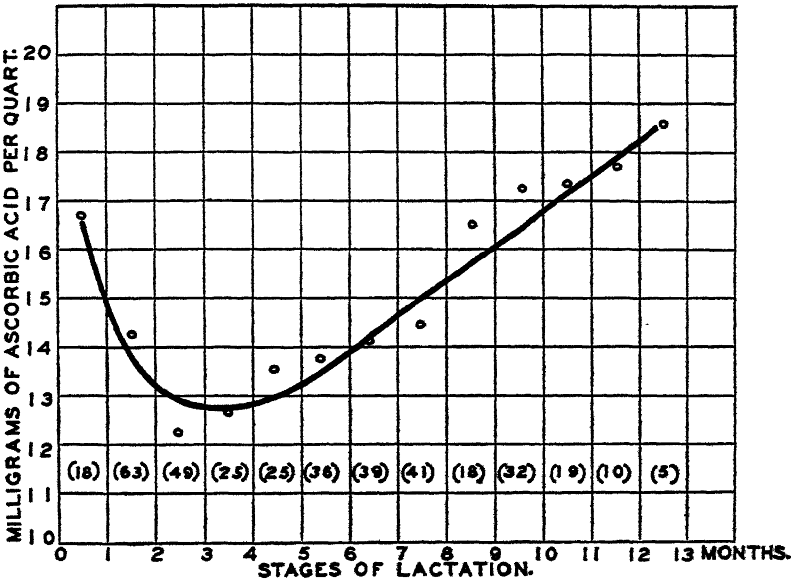

Fig. 1 Showing the effect of the stage of lactation on the ascorbic acid content of the milk produced. The numbers given in enclosure represent the number of samples of milk titrated during the respective periods.

times during the 39-day experimental period. These cows ranged from 3 to 10 years of age and represented all stages of lactation.

It may be observed from the data given in table 1 that the ascorbic acid content of the various milk samples varied with the cow almost as much as it varied with the breed. It may also be observed from the data given in this table that the ascorbic acid content of milk from some cows showed slight variations from period to period. While no explanation is

offered for the latter variation, it seems highly probable that at least part of the variation in the ascorbic acid content of milks produced by cows of the same breed is due to different stages of lactation.

The data presented, while not showing sharp differences in the ascorbic acid content of milks from cows of different breeds, indicate that slight differences do exist in the case of certain breeds. While the average ascorbic acid content of milks from cows of the Ayrshire, Guernsey and Jersey breeds was found to be approximately the same, the average ascorbic acid value of thirty-six samples of milk from Brown Swiss cows was 48 per cent above that obtained for a similar number of milk samples from Holstein cows. This difference in ascorbic acid content could not be explained on the basis of the difference in the volume of milk produced, as the average volume of milk produced by the two breeds during the experimental periods was found to be approximately equal.

In the studies relative to the effect of the stage of lactation on the ascorbic acid content of cow's milk, a total of 410 samples of milk were considered. These samples of milk represented all stages of lactation from post-parturition to the thirteenth month of milk production. As was the case in the studies relating to the effects of breed characteristics, these samples of milk were collected directly from the respective cows and the ascorbic acid content determined at once by titration with a standard solution of the 2–6 dichlorophenolindophenol. In order to conserve space, the data obtained in this phase of the investigation have been arranged in accordance with the stage of lactation of the respective cows and are presented in graphic form.

It will be observed from these data (fig. 1) that the average ascorbic acid content of milks produced by a mixed dairy herd, receiving similar rations, varied widely according to stages of lactation. Eighteen samples of such milk, produced during the first month of lactation, were found to contain an average of 16.7 mg. of ascorbic acid per quart, while forty-nine samples of milk produced during the third month of lactation contained an average of only 12.3 mg. per quart, and

five samples of milk produced during the thirteenth month of lactation contained 18.6 mg. per quart. Samples of milk taken at intermediate stages of lactation gave intermediate ascorbic acid values.

A few samples of colostrum were also titrated and in each case the ascorbic acid content was found to be unusually high. This high ascorbic acid content of colostrum was believed to indicate a certain degree of storage of this substance by the cow during the pre-parturition period. The data presented in figure 1 show, however, that if such a body-store of ascorbic acid does exist it must be limited, since 2 months after parturition the amount of this substance in the milk has dropped to a minimum. After the first 2 months of lactation, the ascorbic acid content of milk is apparently dependent solely upon the ascorbic acid content of the diet of the cow. With a constant intake of ascorbic acid in the diet of the cow and a decrease in milk production such as usually occurs in the later stages of lactation, one might expect milk of higher ascorbic acid content, especially if the storage capacity of the cow for this substance is limited.

A point which is probably worthy of mention in this connection concerns the apparent relation between 'cardboard-flavor' and low ascorbic acid content of milks. It was observed, invariably, that those samples of milk, portions of which had been titrated for ascorbic acid content, which developed cardboard-flavor on standing, decreased in ascorbic acid content coincident with the formation of the off-flavor. Whether cardboard-flavor is due to the degradation products of ascorbic acid or whether those factors instrumental in the formation of cardboard-flavor are also instrumental in the destruction of ascorbic acid was not determined.

SUMMARY

A study has been made concerning the effects of breed characteristics and stages of lactation on the ascorbic acid content of cow's milk. As a result of these studies, the following conclusions are drawn:

1. Cows of the same breed, while receiving similar diets, may produce milks which show wide variations in their ascorbic acid content. Such variations in ascorbic acid content are thought to be due, in part at least, to differences in stages of lactation.

2. Cows of different breeds, while receiving similar diets, produced milks which differed somewhat in their average ascorbic acid values. Of the five breeds studied, the Brown Swiss cows produced milks of the highest ascorbic acid content, while the Holstein cows produced milks of the lowest ascorbic acid value.

3. Stage of lactation appeared to have a more definite effect upon the ascorbic acid content of milk than did breed differences. The ascorbic acid content of milk was found to be relatively high during the early stages of lactation, but decreased to a minimum after about 2 months of lactation, and then increased to a maximum in the later stages of lactation.

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THE EFFECT OF CELLULOSE, HEMICELLULOSE AND LIGNIN ON THE WEIGHT OF THE STOOL: A CONTRIBUTION TO THE STUDY OF LAXATION IN MAN

RAY D. WILLIAMS AND WILLIAM H. OLMSTED

WITH THE ASSISTANCE OF C. H. HAMANN, J. A. FIORITO AND DOROTHY DUCKLES

Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri

TWO FIGURES

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Of late we have been concerned with the physiological effects of the indigestible residue fraction of foods (lignin, cellulose and hemicellulose). In a previous paper (Williams and Olmsted, '35) we demonstrated by a new method that 'crude fiber' is not a chemical entity. Our method, which determines separately lignin, cellulose and hemicellulose, made possible a reinvestigation of: First, the determination of these products in foods containing indigestible residues; second, the fate of each of these fractions when fed to human subjects, and third, the effect of cellulose, lignin and hemicellulose on the bulk of the stool.

As a corollary to our experiment the problem of the utilization of indigestible residues should be attacked. For at least 70 years attempts have been made to determine how much cellulose is broken down in the gastro-intestinal tract of mammals. The problem is of special interest in animal husbandry in that the economy and nutrition of ruminant herbivores is involved. It is estimated (McCance and Lawrence, '29) that a considerable part of the caloric intake of a cow comes from bacterial ferment digestion of residues

in the rumen. The suggestion has been made that man obtains nutriment from intake of indigestible residue in an analogous manner. McCance and Lawrence concluded, however, that from this standpoint the indigestible carbohydrate portion in the human diet was of little importance. The progress of the problems in reference to the utilization of crude fiber has been excellently reviewed by Mangold ('34). For the most part, workers have been unable to surmount the unsuitability of analytical methods. A serious objection to previous work is in the pre-treatment of the materials fed. It is obvious that vigorous chemical treatment, as in preparing Weende crude fiber, profoundly alters its composition and the resultant digestibility data are meaningless when applied to naturally occurring residues. In the light of the foregoing criticism we doubted the validity of the results obtained in one of our studies (Olmsted, Curtis and Timm, '35) of human subjects who were fed pentosan extracted from wheat bran by means of strong alkali. In the present study this doubt is confirmed; our results indicate that a chemical pre-treatment profoundly alters the extent to which indigestible residue disappears from the gut.

So far, it is generally agreed that the end products of carbohydrate residue disintegration in the digestive tract are soluble sugars, fatty acids, gases (methane, hydrogen, carbon dioxide) and alcohols. Symbiotic bacterial enzymes evidently hydrolyze cellulose and hemicellulose into soluble products which either are absorbed as such or are immediately fermented to gases and fatty acids of the lower hydrocarbon series. It seems that the initial reaction is the determinant, for only by means such as Pringsheim ('23) used can intermediary products be isolated. Pringsheim stopped the reaction in an intermediary stage and detected cellobiose (an intermediary between cellulose and reducing sugars-maltose and dextrose) and reducing sugars. Khouvine ('23) isolated from the human gastro-intestinal tract a cellulose splitting bacterium in pure culture. In vivo the case is not as simple. Certainly in this experiment we found a very

small quantity of copper reducing, sugar-like substances and an abundance of volatile fatty acids. For this reason we quantitatively determined volatile fatty acids which are easily determinable end products of indigestible residue breakdown.

Bahrdt and Edelstein ('11) showed that lower volatile fatty acids when fed to dogs increase the volume of the stool. Grove, Olmsted and Koenig ('29) indicated that high carbohydrate diets augment the stool weight more than either high fat or high protein diets. Pediatricians have recognized the efficacy of added sugar in infant feeding to promote laxation. Grove, Olmsted and Koenig further showed that by high carbohydrate diets the stool volatile fatty acids could be tremendously increased; but they also demonstrated that stool volatile fatty acids can be increased in human subjects by simple mineral catharsis. Therefore, it appears that a high volatile fatty acid content in the stool either accompanies an increased stool volume or is the cause of it. There is considerable evidence from our experiment that the relationship is not merely a concomitant one but definitely causal in nature.

EXPERIMENTAL

In outline, our plan was to isolate indigestible residues from naturally occurring sources with as little change as possible in their original compositions (subsequent analysis showed no essential change). We fed these prepared materials to human subjects on a fixed basal diet free from indigestible residue; analyzed the resultant stools for indigestible residue, volatile fatty acids and soluble hydrolyzable reducing substances and noted the subjective effects. We recorded the wet weight of the stool as passed, and from this value subtracted the average (of three) basal period stool weights plus the weight of the residue which had been recovered in the feces. This value we define as the *increment of stool weight.*

1. Preparation of feeding materials

Our object in pre-treatment of the materials was to concentrate the indigestible residue by removal of water, starch, sugars, minerals, oils, fats and resins. By concentration we obtained more nearly homogenous samples in manageable quantities and eliminated substances which might complicate the experiment.

We obtained from the open market carrots, cabbage and canned peas. After grinding in a food chopper to about 20 mesh (dry) each material was suspended in a mechanical washer and washed for 24 hours at 65°C. The washer was of this type: The material was inclosed in a 20-mesh wire gauze cylinder which in turn was suspended in a slightly larger cylinder of sheet metal. The water was forced spirally from the bottom of the inner cylinder and at such a rate that the material was thoroughly mixed and suspended during the washing time.

Wheat, bran, alfalfa leaf meal, corn germ meal, cotton seed hull meal and sugar beet pulp were secured from the mill[1] already ground. These were washed as were the vegetables.

Subsequently each of the materials was quickly air dried at about 40°C. and then extracted with boiling ethyl alcohol in a Soxlet apparatus for 24 hours. The alcohol was evaporated and the materials again air dried. In addition to the materials listed above, cellu flour[2] (40 mesh) and agar agar (20 mesh) were fed without additional preparation, for analysis showed that additional treatment was unnecessary.

Analyses of the materials before and after pre-treatment indicated that the process did not alter the essential composition of the indigestible residue but did remove most of the water, starch, soluble hemicelluloses, sugars, minerals, fats, oils, resins and coloring matter, and thus concentrated the residue. The pre-treatment was not made quantitative but 60 pounds of carrots, 60 pounds of cabbage and 42 cans (no. 2) peas were used to obtain the indigestible residue fed during the vegetable periods.

[1] Courtesy of the Ralston Purina Co., St. Louis, Mo.

[2] A product of the Chicago Dietetic Supply House.

We attempted the preparation of linseed meal and canned corn but found that these necessitated an enzymatic treatment in addition to the process described above. Since their residue composition so closely resembled that of other materials on our list, we did not feed these two products.

2. *Selection of subjects*

We selected three male medical students whose colons by past history and x-ray studies appeared normal but differed in type. Subject 'W' was hyposthenic, subject 'F' was hypersthenic and subject 'H' was intermediary. This selection was made in view of the theory that colonic type influences the coefficient of utilization of indigestible residue and the stool volume.

3. *Basal diets*

All meals were prepared in the metabolism ward kitchen of Barnes Hospital. The foods used were saccharose, strained orange, lemon and grapefruit juices, meats, dairy products, white flour in bread, spaghetti, noodles and jello. *No vegetables or materials containing indigestible residue were fed.* We did not feed charred meats or toast and the crust of bread was removed. This precaution is necessary, for carbon apppears quantitatively in the lignin fraction of stools. Outside the basal diet we allowed only water, ginger ale and small quantities of black coffee (unsweetened). Subsequent analyses of the stools from the basal diet indicated an inconsiderable amount of indigestible residue. (Per week, subject 'F' 1.8 gm.; subject 'W' 1.6 gm.; subject 'H' 2.9 gm.) In our analysis the residue was fractionated into lignin, cellulose and hemicellulose. Although the basal residue represented a very small fraction of the material recovered from a feeding period, it was subtracted from the total recovery. That the basal diet was adequate calorically is indicated by the fact that the subjects' weights were maintained.

4. Method of feeding

The basal diet was fed for 7 days (Monday through Sunday). The total amount of each prepared material was distributed over 6 days (Monday through Saturday); and the stools were collected for 7 days (Tuesday through Monday). The basal diet was repeated precisely to the day and to the meal during each of the feeding periods, and was repeated alone on the sixth and tenth weeks: viz., basal, four feeding periods; basal, three feeding periods; basal, three feeding periods—a total of 13 weeks. As a rule the material was fed as a dry cereal with cream and sugar.

The amount of material fed was determined in this way: In the past the general rule has been to determine the Weende crude fiber value of a material, to feed it irrespective of its chemical composition and to determine, by the same procedure, the crude fiber in the stool. In our previous paper ('35) we indicated the inadequacies of this method, for Weende crude fiber represents a variable fraction of the total indigestible residue; and what becomes of the fraction of residue not determined as crude fiber is not known. Furthermore, it is in no wise true that material which has passed through the gut is as susceptible to chemicals as the original material. We believe that it is more susceptible and that the loss attributed to utilization may have been partially apparent. Certainly we have found that consecutive Weende treatment of crude fiber causes additional losses in the lignin and hemicellulose fractions. Our method of analysis makes possible the fractionation of indigestible residue and the separate following of each component in the gut.

Since we did not know the relative influence of the three components (lignin, cellulose and hemicellulose) on the volume of the stool, but suspected that lignin and cellulose were the important fractions, we kept these two constant and fed 10 gm. of lignin plus cellulose per day with the hemicellulose as the variable. Our results did not bear out this hypothesis, for hemicellulose proved very important not only in its break-

down but also in the hydroscopic properties of residues which we believe to be attributable chiefly to the hemicellulose fraction.

Table 1 presents the analyses of materials quite different in their composition of indigestible residue. The cellulose ranges from 0 per cent to 78 per cent, lignin from 0 per cent to 21 per cent and hemicellulose from 9 per cent to 81 per cent. In table 1 hemicellulose is divided into two fractions. This

TABLE 1

Analysis of materials fed

MATERIALS ADDED TO THE BASAL DIET	WHEAT BRAN	ALFALFA LEAF MEAL	CARROTS	CORN GERM MEAL	COTTON SEED HULL	SUGAR BEET PULP	CANNED PEAS	CABBAGE	AGAR AGAR	CELLU FLOUR
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Cellulose	16.9	32.5	23.2	15.8	19.4	34.2	35.0	29.5	0.0	78.8
Lignin	7.8	15.0	3.4	2.4	20.8	2.5	1.7	2.6	0.0	0.0
Hemicellulose (S)	35.2	15.5	20.7	28.9	30.9	20.4	8.8	19.8	81.0	16.9
Hemicellulose (L)	0.0	3.7	8.1	1.9	0.6	8.8	2.1	8.5	....	0.0
Starch	6.6	2.5	0.7	10.9	3.2	1.8	13.6	0.0	0.0	0.0
Protein (N×6.25)	12.3	12.4	7.8	18.6	7.3	8.5	16.5	10.8	1.6	0.0
Moisture (110°)	8.9	6.5	11.1	7.5	8.2	7.9	7.5	10.0	13.5	3.0
Ash (red heat)	4.4	2.3	5.8	5.5	1.8	2.3	2.2	3.8	3.4	0.1
Soluble in alcohol-benzene	3.5	9.0	15.2	7.2	7.0	9.8	9.7	12.5	0.0	0.0
Total	95.6	99.4	96.0	98.7	99.2	96.2	97.1	97.5	99.5	98.8

(L) Soluble in solutions pH 8.
(S) Insoluble in solutions pH 8.

division is necessitated by the fact that the term hemicellulose merely designates a group, some of whose members are resistant to concentrated alkali and others are susceptible to the dilute alkali (pH 8) which is used in our method of analysis. This latter fraction is variable but usually quite small, and is called the labile fraction (L). Bran and alfalfa leaf meal have the same ratio of cellulose to lignin; but for 10 gm. of lignin plus cellulose, bran has four times as much hemicellulose (table 2). Carrots and corn germ meal have the

same cellulose to lignin ratio; but for 10 gm. of cellulose plus lignin, corn germ meal has twice as much hemicellulose. From these two pairs one should be able to determine the influence of lignin on the breakdown of cellulose and hemicellulose and the influence of hemicellulose on stool volume. Cotton seed hull meal is an example of a material with an exceedingly low ratio of cellulose to lignin; and if lignin influences the breakdown of cellulose and hemicellulose, this material should indicate the fact. Agar agar is an example of a material containing only hemicellulose. Cellu flour is an

TABLE 2

Amounts of materials fed to each subject during each 6-day period

MATERIALS ADDED TO THE BASAL DIET	MATERIAL FED	LIGNIN	CELLULOSE	HEMICELLULOSE	TOTAL INDIGESTIBLE RESIDUE
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Wheat bran	243.0	18.9	41.1	86.0	146.0
Alfalfa leaf meal	126.5	19.0	40.9	19.6	79.5
Carrots	226.1	7.7	52.3	46.8	106.8
Corn germ meal	328.9	7.8	51.9	95.0	154.8
Cotton seed meal	149.0	31.0	28.9	46.0	105.9
Sugar beet pulp	163.5	4.1	55.9	33.4	93.4
Canned peas	163.8	2.7	57.3	14.4	74.5
Cabbage	210.6	5.5	54.5	41.5	101.5
Agar agar	99.0	0.0	0.0	80.2	80.2
Cellu flour	84.0	0.0	60.5	11.2	71.7

example of a material prepared analogously to Weende crude fiber or filter paper, materials which have long been fed to increase the stool weight. Table 2 gives the exact amounts of materials fed. Although the total amounts of materials fed varied from 84 to 329 gm., for 6-day periods, the amounts of cellulose plus lignin were in all instances 60 gm.

5. *Collection of stools*

Each stool was collected free from urine, weighed as passed; the wet weight was multiplied by 5 and this number of cubic centimeters of distilled water added. The stool was then thoroughly mixed by a mechanical mixer, put in a fruit

jar and steam sterilized (15 pounds steam pressure, 30 minutes), sampled aseptically and the sample analyzed immediately or stored under toluol at 6 to 10°C. Since the feeding was discontinued Saturday at 9 A.M. and the last stool collected at 9 A.M. Monday, analysis of the stools showed that essentially 100 per cent of the material to pass the gut came through in that time (48 hours). We did not use charcoal or carmine to mark the stools; but during the first five periods we analyzed the stools on the third, sixth and seventh days of stool collection. Since the results of these periods showed nothing to be gained by so many separate analyses, we pooled the 7-day stools of each subject and made one analysis in triplicate of each period.

6. *Methods of analyses*

Indigestible residue in the materials fed[3] and in the stools was determined by the method of Williams and Olmsted ('35) with the exception that, since the materials were already graded to 20 mesh, no additional comminution was neceessary.

Volatile fatty acids were determined by the method of Olmsted et al. ('29) on stool filtrates prepared by the procedure of Steiner, Urban and West ('32) using ferric sulphate and Lloyd's reagent as the precipitating agent. We used 60 cc. stool suspension (1:6) and 25 cc. ferric sulphate with a final dilution of 250.

Soluble sugar or sugar-like substances were determined on the West filtrate both before and after hydrolysis (1.5 N H_2SO_4; 2 hours; 100°C.) by the technic of Shaffer and Somogyi ('35) and the fermentation procedure of Somogyi ('28). Exploratory analyses indicated that the copper re-

[3] Agar agar cannot be analyzed by our usual procedure. Instead of the enzymatic pre-treatment followed by strong acid hydrolysis we hydrolyzed it by 2.5 N. H_2SO_4, 100°C., 3 hours. Subsequent determination of reducing sugars follows our original method. Agar stools were thus hydrolyzed and then precipitated by the West method (Steiner, Urban and West, '32) before analysis of the reducing sugars in the hydrolysate. Basal period stools were treated identically and their very small residue values subtracted from the agar period residues.

ducing value of the West ferric sulphate reagent filtrate was lower than that of either the mercuric sulphate (West, Scharles and Peterson, '29) or the mercuric-ferric sulphate (E. S. West, unpublished) reagent filtrates and that the volatile fatty acids were identical in all three. We concluded, therefore, that the high acid concentration of the mercuric sulphate reagent hydrolyzed some of the more labile residue. With the ferric sulphate reagent combined with Lloyd's reagent filtrate we found only traces of reducing substances either before or after acid hydrolysis of the filtrate.

Criteria of increased stool volume: We agree with Cowgill and Anderson ('32) that the impression of the subject is an important criterion; and we attempted to rate our materials accordingly. Most certainly the volume of the stool, its dry weight or its wet weight are not the only criteria. Our subjects reported considerable difficulty in passing stools from such materials as alfalfa leaf meal, cotton seed hull meal, cellu flour, and to a certain extent, wheat bran. Here the stool weight and stool volume were quite high but the feces were dry, hard and often flecked with blood. An objective rating, which coincides remarkably well with the subjective rating, is what we term the increment in stool weight which we have already defined.

Inasmuch as the results of this study revealed a direct and unexpected relation between the increment in the stool weight and the *percentage disappearance* of ingested food residue from the gut instead of *percentage recovery* in the stool, the data presented in tables 3 and 4 and figure 2 pertain to disappearances rather than recoveries.

RESULTS

The results are given in tables 3, 4, 5 and 6.

Table 3 gives the percentage disappearance of each of the three fractions of indigestible residue (lignin, cellulose and hemicellulose) for each of the three subjects. These observations can be made: 1) The percentage disappearance of any one material is strikingly uniform for the three subjects.

2) In general, the magnitude of the percentage disappearance is in this order: Hemicellulose, cellulose and lignin. The conclusion is based on only those materials containing large percentages of lignin. 3) The percentage of lignin in naturally occurring materials profoundly influences the percentage disappearance in the human gut. This is most striking when cotton seed hull meal (high in lignin) is compared with other substances lower in lignin; or when the group high

TABLE 3

Percentage disappearance of materials fed

MATERIALS ADDED TO THE BASAL DIET	SUBJECT F				SUBJECT W				SUBJECT H			
	Lignin	Cellulose	Hemicellulose	Total indigestible residue	Lignin	Cellulose	Hemicellulose	Total indigestible residue	Lignin	Cellulose	Hemicellulose	Total indigestible residue
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Wheat bran	10.5	31.6	33.6	30.0	4.0	24.0	32.0	26.2	16.0	33.0	39.0	34.2
Alfalfa leaf meal	5.5	11.2	9.0	9.5	0.0	12.5	0.0	7.0	4.0	13.0	7.6	10.0
Carrots	55.8	72.5	88.9	79.4	50.6	62.3	80.0	69.2	54.6	65.6	84.6	73.1
Corn germ meal	44.9	55.4	62.5	59.3	55.0	62.0	67.4	64.9	41.0	53.4	57.9	55.6
Cotton sead hull meal	23.5	23.9	37.4	29.6	3.0	3.2	14.2	7.2	10.0	25.0	39.5	17.5
Sugar beet pulp	36.5	41.8	88.3	55.0	17.0	62.8	90.4	70.7	36.6	61.4	88.0	69.8
Canned peas	63.6	40.0	79.9	48.4	66.6	44.8	86.2	53.4	39.3	49.0	86.8	55.8
Cabbage	72.7	61.3	79.0	69.2	54.5	43.5	80.5	59.3	69.1	59.2	80.2	68.4
Agar agar	....	....	65.5	65.5	....	....	60.2	60.2	....	....	55.2	55.2
Cellu flour	....	10.9	47.7	15.9	....	10.0	28.0	13.0	....	0.0	10.8	2.0

in lignin (cotton seed hull, wheat bran, alfalfa leaf) is compared with the group low in lignin (carrots, corn germ, peas, beet pulp, cabbage). This confirms the original postulate of Waentig and Gierisch ('18). 4) The slight disappearance of cellu flour would seem to indicate that processing remarkably lowers the percentage disappearance. If energy values and utilization are considered, it would seem to be indicated that our human subjects derived little nutritive value from the indigestible residue fed. We fed very nearly the maximum amount of indigestible residue, for with certain materials

there was at least one exceedingly large stool per day. Larger amounts would have caused diarrhea. With corn germ meal 89 gm. of cellulose plus hemicellulose disappeared in 6 days. This represents 14.5 gm. per day from which could be obtained only a very small fraction of the 2300 to 2800 Cal. daily needs of our subjects.

Since it has been suggested that volatile fatty acids may be a stimulus to the gut, we attempted to correlate the increment (over basal) in stool volatile fatty acids with the increment in stool weight and the cellulose plus hemicellulose disappearing. Table 4 presents in detail the data of the three subjects. Attention is again called to the close agreement between the three subjects.

Table 5, a composite of the data from the three subjects, relates the increment in stool weight, the amount of indigestible residue fed and the residue remaining in the stools. Figure 1 graphically presents the data of table 5.

These observations can be made: 1) The increment in stool weight does not correspond to the amount of residue fed. 2) An inverse relationship exists between the increment in stool weight and the material recovered in the stool, i.e., the less material recovered the greater the increment in the stool weight. It would appear, therefore, that the amount disappearing from the gut determines the true increase in stool volume.

It may be objected that the increment in stool weight represents merely water absorbed by the residue not disappearing from the gut, i.e., the hydroscopic properties of the residue. However, column 5 of table 5 would answer this objection in that, for instance, each gram of agar agar would have increased the stool weight 20 gm., each gram of cabbage 18 gm., each gram of carrots 19 gm., and each gram of sugar beet pulp 12 gm. To us this hypothesis seems untenable and we conclude that some factor in addition to the hydroscopic properties of the stool residue must account for the increment in stool weight.

TABLE 4

Increment in stool weight related to increment in volatile fatty acids and cellulose plus hemicellulose disappearing

MATERIAL ADDED TO BASAL DIET	SUBJECT F					SUBJECT W					SUBJECT H				
	Increment stool volatile fatty acids (1.0 N alkali)	Increment stool weight	Cellulose plus hemi-cellulose disappearing			Increment stool volatile fatty acids (1.0 N alkali)	Increment stool weight	Cellulose plus hemi-cellulose disappearing			Increment stool volatile fatty acids (1.0 N alkali)	Increment stool weight	Cellulose plus hemi-cellulose disappearing		
			Hemi-cellulose	Cellulose	Total			Hemi-cellulose	Cellulose	Total			Hemi-cellulose	Cellulose	Total
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
Wheat bran	89.4	575	28.9	13.1	41.0	98.7	578	27.2	9.9	37.1	116.0	373	33.5	13.4	46.9
Alfalfa leaf meal	12.4	182	1.8	4.6	6.4	36.7	318	0.0	5.2	5.2	34.5	221	1.5	5.4	6.9
Carrots	104.4	700	41.6	37.9	79.5	109.7	589	37.4	32.6	70.0	77.0	336	39.6	34.3	73.9
Corn germ meal	98.4	590	59.4	28.8	88.2	84.2	563	64.0	32.2	96.2	148.0	581	55.1	27.7	82.8
Cotton seed hull meal	7.9	104	17.2	6.9	24.1	52.7	123	6.6	0.2	6.8	24.0	66	8.2	7.3	15.5
Sugar beet pulp	178.4	662	29.5	23.4	52.9	75.7	372	30.2	35.2	65.4	40.0	171	29.4	34.3	63.7
Canned peas	60.9	114	11.5	22.8	34.3	34.7	255	12.4	25.5	37.9	38.8	134	12.5	28.1	40.6
Cabbage	136.4	647	32.8	33.4	66.2	158.7	781	33.4	23.7	57.1	110.5	449	33.3	32.3	65.6
Agar agar	60.4	835	53.4	....	53.4	98.0	719	48.3	....	48.3	110.5	445	44.3	....	44.3
Cellu flour	3.4	223	5.3	6.3	11.6	9.7	119	3.1	6.2	9.3	(—) 2.5	(—) 63	1.2	0.2	1.4

TABLE 5

Relationship of increment in stool weight to residue fed and residue remaining in the stool

MATERIALS ADDED TO THE BASAL DIET	INDIGESTIBLE RESIDUE FED (1)	INDIGESTIBLE RESIDUE REMAINING IN STOOL (2)	INCREMENT IN STOOL WEIGHT (3)	RATIO INCREMENT STOOL WEIGHT TO WEIGHT OF RESIDUE FED (3)/(1) (4)	RATIO INCREMENT STOOL WEIGHT TO INDIGESTIBLE RESIDUE IN STOOL (3)/(2) (5)
	gm.	*gm.*	*gm.*		
Wheat bran	146.0	102.0	475	3.25	4.65
Alfalfa leaf meal	79.5	72.5	240	3.04	3.00
Carrots	106.8	27.8	541	5.62	19.30
Corn germ meal	154.8	62.0	578	4.58	9.34
Cotton seed hulls	105.9	86.6	97	2.06	1.13
Sugar beet pulp	93.4	32.5	401	4.92	12.30
Canned peas	74.5	35.3	167	3.27	4.77
Cabbage	101.5	34.9	625	6.91	17.90
Agar agar	80.2	32.9	666	8.75	20.80
Cellu flour	71.7	64.2	93	2.34	1.45

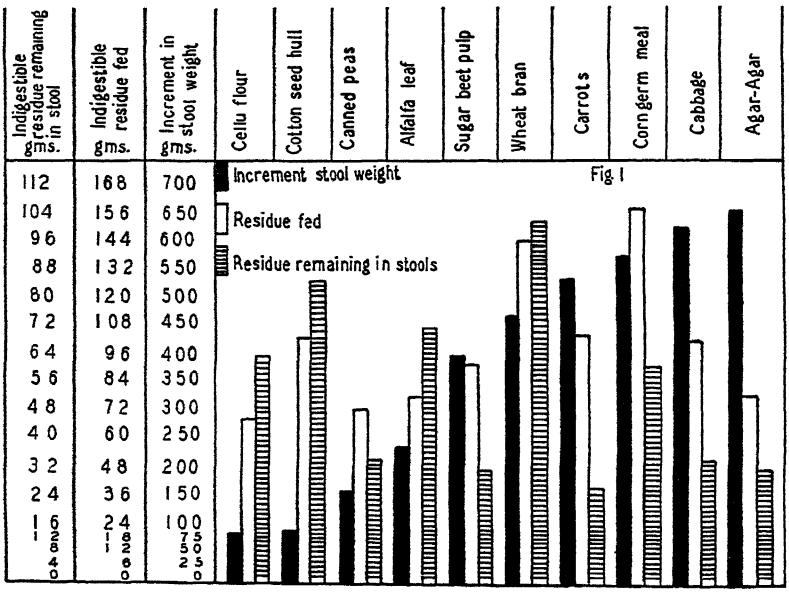

Figure 1

Table 6, a composite from the data of table 4, relates the increment in stool weight, the cellulose plus hemicellulose disappearing and the increment in stool volatile fatty acids. The subjective estimation of the relative values of the materials is also correlated. Figure 2 presents graphically the relationships.

It will be observed that the values of the residue breakdown and the increment in stool volatile fatty acids roughly parallel the increment in stool weight. This then would seem to indicate that breakdown of residue is the important factor; and, since the increment in volatile fatty acids so closely parallels the disappearance of cellulose and hemicellulose, one might reasonably conclude that the acids come from the breakdown of residue and in turn are responsible for the increment in stool weight. If the residue remaining in the stool is very hydroscopic, for instance agar agar, one might expect the increment in stool weight to be more than commensurate with the breakdown of residue and the increment in volatile acids. This may explain the increment in stool weight in the case of agar agar which appears to be greater than the breakdown

TABLE 6

Increment in stool weight related to hemicellulose plus cellulose disappearing and to increment in stool volatile fatty acids

MATERIALS ADDED TO THE BASAL DIET	INCREMENT IN STOOL WEIGHT	HEMICELLULOSE PLUS CELLULOSE DISAPPEARING			INCREMENT IN STOOL VOLATILE FATTY ACIDS (1.0 N ALKALI)	ESTIMATION BY SUBJECTS OF RELATIVE LAXATION VALUES (1 LEAST; 10 MOST)
		Hemi-cellulose	Cellulose	Total hemi-cellulose plus cellulose		
	gm.	*gm.*	*gm.*	*gm.*	*cc.*	
Wheat bran	475	29.8	11.4	41.2	101.3	5
Alfalfa leaf meal	240	1.1	5.0	6.1	27.8	1
Carrots	541	39.5	34.9	74.4	97.0	9
Corn germ meal	578	59.5	29.5	89.0	110.2	8
Cotton seed hull	97	10.6	4.8	15.4	28.2	3
Sugar beet pulp	401	29.7	30.9	60.6	98.0	7
Canned peas	167	12.1	25.4	37.5	44.8	4
Cabbage	625	33.1	29.8	62.9	135.2	10
Agar agar	666	48.6	....	48.6	89.6	6
Cellu flour	93	3.2	4.2	7.4	3.5	2

of residue and the production of volatile fatty acids would lead one to expect.

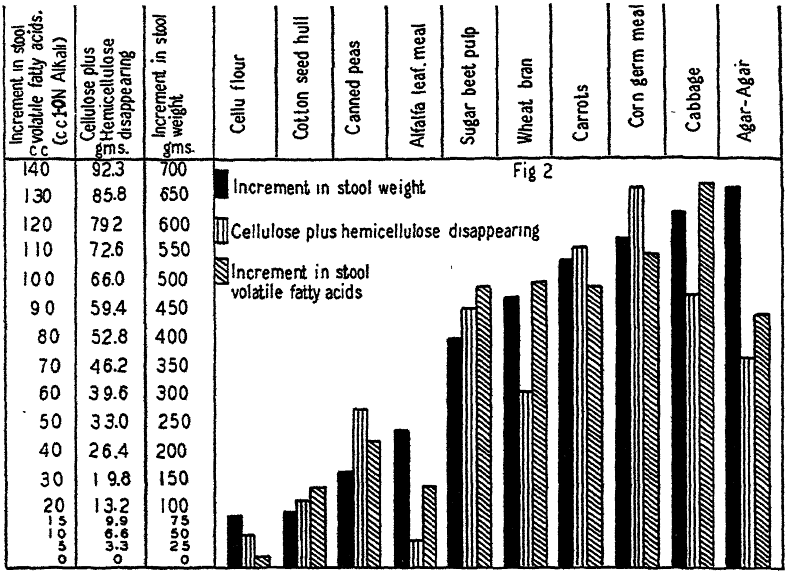

Figure 2

SUMMARY AND CONCLUSIONS

1. The indigestible residues (lignin, cellulose and hemicellulose) found in ten food substances of widely varying sources were concentrated by simple procedures which would not alter the essential composition of the residue. These were added to a non-residue diet and fed to three human subjects.

2. By analysis of the feces it was found that hemicellulose disappeared in larger amounts than cellulose and that lignin disappears least.

3. When there was a high percentage of lignin in the residue, less hemicellulose and cellulose disappeared from the gut.

4. A comparison of the stool weights after feeding of these residues seemed to indicate that the amount of cellulose and hemicellulose disappearing during the passage through the

human gut influenced the volume of the feces more than the amount of residue fed or the amount recovered in the feces.

5. In general, the quantity of stool volatile fatty acids was greatest when a residue disappeared most during its passage through the gastro-intestinal tract.

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THE VITAMIN G COMPLEX

I. THE NON-IDENTITY OF RAT DERMATITIS DUE TO VITAMIN B_6 DEFICIENCY AND THE DERMATITIS OF HUMAN PELLAGRA

WILLIAM JOHN DANN
BEIT MEMORIAL RESEARCH FELLOW

Department of Physiology and Pharmacology, Duke University, Durham, North Carolina

(Received for publication January 18, 1936)

In this study an examination has been made of the position of the newly discovered components of the vitamin G(B_2) complex in relation to the etiology and treatment of pellagra. Ever since Goldberger and Lillie ('26) reported the discovery of a 'pellagra-like' condition in the rat, apparently traceable to the same dietary deficiency as human pellagra, the hypothesis that vitamin G(B_2) and the pellagra-preventive factor are one and the same entity has been widely accepted. With the aid of this hypothesis as a starting point many studies have been made, of which a considerable number lead to conclusions at variance with the idea that human pellagra is caused by a deficiency of vitamin G(B_2). This has led to much confusion, one result of which is that the vitamin G deficiency hypothesis of the causation of pellagra has been rejected by many workers. This hypothesis was nevertheless accepted by the writer because it appeared to be at least as conformable with past observations on pellagra as any of the other hypotheses which have been advanced.

Recently, however, our knowledge of vitamin G has been greatly extended by the work of György, Kuhn and Wagner-Jauregg ('33) who have shown that vitamin G is not a single substance, but a mixture of lactoflavin with a second substance

which György has since called vitamin B_6. György ('34, '35) has examined the nature and distribution of this second substance and has shown that it is the factor whose absence from the diet will induce the pellagra-like dermatitis in the rat. His observations have been substantially confirmed by Chick, Copping and Edgar ('35) and by Harris ('35). This demonstration that vitamin G is made up of two factors, one of which controls the characteristic dermatitis of the rat,[1] appeared to open up the possibility that some of the confusion existing in this field might be cleared away. The first step necessary for a clarification of the position was to determine whether either or both of the newly discovered components of vitamin G possess pellagra-preventive activity. In the following section an account is given of experimental and clinical work directed to that end.

EXPERIMENTAL

1. The curative action of cornmeal in 'rat pellagra.' In a preliminary examination of the effect of white maize meal on the specific dermatitis of vitamin B_6 deficiency in the rat, three rats with severe symptoms were each given 1 gm. daily of white maize meal and all three recovered completely in 3 to 4 weeks. This result is of key importance in determining whether vitamin B_6 plays any part in the etiology of human pellagra and the observation was accordingly repeated and extended as follows. Sixteen rats at the age of 29 days were placed in screen bottom cages and given the vitamin B-free diet of György (vitamin B-free casein, Harris, 18 per cent; rice starch 68 per cent; butterfat 9 per cent; cod liver oil 1 per cent; salt mixture, McCollum's, 4 per cent) ad libitum. For the first 30 days of the experiment no supplement was added to this diet, but from the thirtieth day onward each

[1] Throughout this paper the term rat dermatitis is used to designate the condition termed 'symmetrical pellagra-like dermatitis' by György ('35) or 'florid dermatitis' by Chick, Copping and Edgar ('35). The nomenclature used is that employed by György; namely, vitamin $G(B_2)$ for the sum of the heat stable fractions of the vitamin B complex, and vitamin B_6 for the fraction which controls the rat dermatitis. Lactoflavin is throughout referred to by that name.

rat was given daily 10 γ of crystalline vitamin $B(B_1)$ and was given twice weekly 20 γ of lactoflavin. On this regimen the rats failed to gain weight, developed the specific dermatitis of vitamin B_6 deficiency and appeared listless and in poor condition generally.

Two matched groups of six rats each were then chosen from the sixteen, three in each group having a moderately severe dermatitis, and three in each group having a very severe dermatitis. Each member of the first group was given 1 gm. daily of white maize meal and each member of the second group 1 gm. daily of yellow maize meal in addition to the vitamin B and lactoflavin. The remaining four rats served as negative controls and received no other dietary supplement in addition to the vitamin B and lactoflavin. The results of this experiment are summarized in table 1.

As will be seen from the table the control rats all lost weight and died with very severe dermatitis after an average survival period of 41 days (commencing from the day on which the experimental animals were first dosed). On the other hand those dosed with 1 gm. daily of either white or yellow maize meal all lived through a dosing period of 35 days and gained weight. All rats dosed with yellow maize meal were cured of dermatitis during this period and all except one were in good condition at the close of the experiment. This rat (♂ 00(3)) showed a weight increase above the average and was normally active, but its coat was in bad condition, being extremely thin all over the body. Of the six rats dosed with white maize meal five were cured of their dermatitis and were in good condition at the end of the experiment. The remaining rat (♂ 10(3)) was in poor condition, suffering from an almost complete generalized alopecia, and it still had a slight dermatitis.

From these data it is concluded that 1 gm. of maize meal, whether yellow or white, contains sufficient vitamin B_6 to bring about rapid cure of the characteristic dermatitis of 'rat pellagra' even when it is severe.

TABLE 1

Summary of test of the vitamin B_6 activity of maize meal

A. Rats treated with white and yellow maize meal

RAT	WEIGHT AT 30 DAYS OLD, GRAMS	CONDITION WHEN FIRST DOSED		DOSE	CONDITION AFTER 35 DAYS' DOSING			WEIGHT INCREASE DURING DOSING PERIOD, GRAMS
		Weight, grams	Dermatitis		Weight, grams	Dermatitis	General	
♂ 02(2)	59	46	+++	1 gm. white maize meal	72	0	Good	26
♂ 10(3)	54	44	+++	1 gm. white maize meal	57	+	Poor	13
♂ 01(5)	52	62	++	1 gm. white maize meal	91	0	Good	29
♀ 10(6)	49	49	++	1 gm. white maize meal	71	0	Good	22
♀ 11(6)	46	42	++	1 gm. white maize meal	60	0	Good	18
♀ 02(6)	44	42	+++	1 gm. white maize meal	62	0	Good	20
♀ 00(1)	65	73	+++	1 gm. yellow maize meal	99	0	Good	26
♂ 00(3)	59	49	+++	1 gm. yellow maize meal	77	0	Poor	28
♂ 10(5)	51	62	++	1 gm. yellow maize meal	89	0	Good	27
♂ 11(5)	52	54	++	1 gm. yellow maize meal	80	0	Good	26
♀ 20(6)	44	44	+++	1 gm. yellow maize meal	62	0	Good	18
♀ 21(6)	49	40	++	1 gm. yellow maize meal	54	0	Good	14

B. Control rats

RAT	WEIGHT AT 30 DAYS OLD, GRAMS	CONDITION WHEN EXPERIMENTAL ANIMALS WERE FIRST DOSED		SURVIVAL PERIOD, DAYS	CONDITION AT DEATH		LOSS OF WEIGHT, GRAMS
		Weight, grams	Dermatitis		Weight, grams	Dermatitis	
♂ 20(2)	69	53	+	45	42	+++	11
♀ 10(3)	50	60	++	39	46	+++	14
♂ 21(5)	46	50	++	41	39	+++	11
♀ 20(5)	50	42	+	38	35	+++	7

All rats received the György diet and supplements of pure vitamin B(B_1) and lactoflavin.

\+ indicates slight dermatitis.

++ indicates severe dermatitis.

+++ indicates very severe dermatitis.

2. The incidence of 'rat pellagra' in relation to light. The results reported in the preceding section suggest that vitamin B_6 which cures the dermatitis characteristic of 'rat pellagra' is not identical with the pellagra preventive vitamin postulated by Goldberger. Evidence was therefore sought which would throw light on the relationship of the dermatitis of 'rat pellagra' to the characteristic dermatitis of human pellagra.

To this end forty-nine rats were divided into two groups, one of which was kept in the light and the other in the dark. The animals of both groups were housed in similar cages and both were given the basal diet described by György. In addition to the basal diet supplied ad libitum each rat in each group was given daily by mouth 10 γ of crystalline vitamin B and twice weekly 20 γ of lactoflavin by mouth. The larger group of thirty-seven rats was kept in a light room with windows on the east, south and west sides. Direct sunshine fell several hours of most days of the summer upon these windows, but the rat cages were sufficiently far from the windows to be out of the direct sunshine. The smaller group of twelve rats was kept in a photographic darkroom protected by a maze so that daylight did not penetrate it. A very dim artificial light was maintained throughout the period of experiment by keeping a shaded 40 watt electric lamp burning; arranged in such a way that the light was all thrown on to a small area of the wall away from the neighborhood of the animals. This supplied a general intensity of illumination just great enough to enable one to see one's way about. An overhead electric lamp supplying a stronger light was used while feeding, dosing or weighing the rats.

The incidence of rat 'pellagra' among the rats of the two groups was found to be similar as can be seen from table 2 where the data are summarized. A similar proportion of rats developed the dermatitis in each of the two groups and the average time on the deficient diet before symptoms appeared was almost the same for each group. In each group a number of the rats remained free from dermatitis and grew through-

out the period of feeding on the B_6 deficient diet; an example of refection.

3. Clinical experience with maize meal and with lactoflavin. By the courtesy of Dr. David T. Smith and Dr. J. M. Ruffin of the department of medicine, Duke University, I am able to include the following case reports made by them upon patients seen in Duke Hospital. Ruffin and Smith ('34) have elaborated a fully controlled technic for testing the pellagra-curative action of foodstuffs and special preparations. The patient on entering the hospital is placed on a diet which is arranged to be deficient in vitamin G but satisfactory in all other respects. After 4 or 5 days the patient's sensitivity toward light is determined and if it is found to be super-

TABLE 2

The effect of illumination on the incidence of rat dermatitis

GROUP	TOTAL RATS IN GROUP	ILLUMINATION OF QUARTERS	RATS DYING WITHOUT DERMATITIS	RATS SHOWING REFECTION	RATS DEVELOPING DERMATITIS	AVERAGE NUMBER OF DAYS ON DEFICIENT DIET BEFORE DERMATITIS DEVELOPED
1	37	Strong daylight	4	6	27	96 (standard deviation 11.7)
2	12	Darkroom	1	2	9	98 (standard deviation 11.3)

normal (characteristic of pellagra) the food or preparation to be tested for pellagra-curative activity is added to the basic diet. If the material is active, then the patient will show rapid improvement and after the lapse of 7 to 10 days his light sensitivity is again examined. If it has returned to normal, then a clear-cut case showing the activity of the material under test will have been obtained. But if the material is inactive, the patient's condition will not improve, and his light-sensitivity on re-examination will again be supernormal. He is then given a known pellagra curative (Valentine's Liver Extract) and if his recovery follows during the ensuing 7 to 10 days and his light sensitivity returns to normal, clear-cut evidence showing the inactivity of the test material will have been obtained.

By means of this technic it is possible to determine finally the pellagra-curative activity of a dietary supplement by means of a few cases, provided that the routine of the test can be rigorously adhered to. It frequently happens, however that the routine must be modified in order to avoid danger to the life of the patient or for other reasons, and the result of the test can then only be taken as an indication, but not as proof positive of the activity or inactivity of the test material.

TABLE 3

Standard basic diet, no. 2

ARTICLE	QUANTITY	PROTEIN, GRAMS	FAT, GRAMS	CARBOHYDRATE, GRAMS	MINERALS, GRAMS			CALORIES
					Ca	P	Fe	
Corn meal	92 gm.	8.3	2.0	69.0	0.011	0.1225	0.0006	
Cane syrup	105 gm.	....	....	89.2	....	....	....	
Flour	111 gm.	12.5	1.2	83.4	0.022	0.1030	0.0010	
Lard	81 gm.	....	81.0	....	....	....	....	
Rice	25 gm.	2.0	0.1	19.6	0.0023	0.0240	0.0002	2890.0
Field peas	90 gm.	19.2	1.4	54.6	0.0756	0.0760	0.0052	
Hominy grits	51 gm.	4.3	0.3	40.6	0.0056	0.0734	0.0005	
Fat salt pork	60 gm.	1.1	51.3	0.0	0.0011	0.0115	0.0001	
Cod liver oil	90 ml.	....	80.0		....	0.0117	....	720.0
Ascorbic acid	90 mg.	0	0		0	....	....	
Iron ammonium citrate	6 gm.	0	0		0	0	1.02	
Calcium gluconate	6 gm.	0	0		0.5580	0	0	
Cheese	60 gm.	17.4	21.6		0.5586	0.4098	0.0007	264.0
Total		64.8	238.9	356.40	1.2387	0.8319	1.0283	3874.0

The basic diet used is constituted as shown in table 3. It will be seen that it contains 92 gm. of maize meal (white) and 51 gm. of hominy grits. Thus the patient receives 143 gm. daily of white maize in these two forms and this exerts no curative effect on the pellagra. Three pellagrins were given

treatment with lactoflavin during the summer of 1935 as described below:

Case 1. (No. 53,890 S. T. A colored female, age 34, was admitted to the hospital June 12, 1935, presenting a typical picture of pellagra, with a rash over the hands, a sore tongue and diarrhea of 4 weeks' duration. She was given the Standard Basic Diet no. 2 (shown in table 3) and commencing on the day after admission a solution of lactoflavin containing 0.5 mg. per ml. was administered subcutaneously, 2 ml. the first day, 8 ml. the second and 4 ml. daily thereafter for 6 days. During this time she failed to improve; the anorexia continued; the diarrhea and tongue became much worse. As no improvement was observed after 8 days of this treatment the lactoflavin was discontinued and the patient was given daily 90 cc. by mouth of Valentine's Liver Extract which had been shown previously by Ruffin and Smith ('34) to be effective in the treatment of pellagra. After 3 days she began to improve and within 5 days she was apparently entirely well.

Case 2. (No. 53,398) J. C. A white male, age 39, was admitted to the hospital June 3, 1935, with a marked diarrhea, a rash over both hands, and a red beefy tongue. He was somewhat disoriented. He was given the Standard Basic Diet no. 2 and observed over a period of 5 days. During this time the tongue remained the same and the diarrhea continued unabated.

Beginning June 7th, 15 ml. of a preparation of liver (no. 266 —a fraction of liver which subsequent studies have indicated to be of little or no value in the treatment of pellagra) was given subcutaneously daily for 10 days. After the second day the tongue was definitely better but the diarrhea was unaffected. Since only slight improvement in the general condition was noted after 5 days, the treatment was supplemented by 8 ml. of lactoflavin daily, administered subcutaneously, for 7 days. No definite change was noted at the end of this period.

On June 17th, the right hand and arm were exposed to direct sunlight for 30 minutes and for 45 minutes the following day. No rash was observed but the tongue became very red and sore. The lactoflavin and liver fraction (no. 266) were discontinued and the patient given 90 ml. of Valentine's Liver Extract by mouth. The tongue presented a normal appearance after 4 days of this treatment and there was noted an improvement in his general condition although the diarrhea remained unchanged at time of his discharge, 7 days after instituting the liver therapy by mouth.

Case 3. (No. 31,387). A white woman, age 43, was admitted to the hospital on May 21, 1935. On a previous admission, she had been found to have amyotrophic lateral sclerosis. Her present illness began 4 weeks before admission with a rash over the hands and face and diarrhea following exposure to sunlight.

She was given the Standard Basic Diet no. 2 and observed for a period of 7 days, during which time her diarrhea became worse. On the eighth and ninth days (May 27th and 28th) her right arm and hand were exposed to direct sunlight for 30 and 60 minutes, respectively. Immediately following this she developed nausea and vomiting; the tongue became worse and the diarrhea increased. Lactoflavin, 4 ml. subcutaneously, was given daily for the next 3 days during which time she became progressively worse. On June 1, it was apparent that she was critically ill and was given large amounts of active liver extract by mouth and glucose intravenously. The patient did not respond to treatment and died on June 2nd.

DISCUSSION

Perhaps the most significant of the observations recorded above is the finding that dermatitis can be induced in rats kept in darkened rooms and given a vitamin B_6 deficient diet. Examination of the figures showing incidence of the dermatitis among the rats kept in a darkened room and those kept in strong summer daylight suggests at once that light plays no part in the production of the lesions; and this conclusion is supported by the observation that among the rats developing dermatitis the average time on the deficient diet before the dermatitis appeared was almost the same for the two groups.

On the other hand the clinical experience of Ruffin and Smith ('35) has shown that the dermatitis of the exposed surfaces of human pellagrins is closely dependent upon the action of sunlight on the patient's skin. Thus there is a sharp distinction between the immediate cause of the dermatitis of the rat and the cause of the dermatitis of human pellagra. A dietary deficiency apparently leads immediately to the production of dermatitis in the rat, but in the human the dermatitis is only produced by the agency of light after

dietary deficiency has played its part. This constitutes an important dissimilarity between the rat dermatitis and human pellagra and affords another reason for reviewing with caution past attempts at tracking down the pellagra preventive factor by means of rat feeding experiments. Since much of the past work was done under the assumptions that vitamin G is a single entity and that the rat dermatitis is strictly analogous to human pellagra, its conclusions may be invalidated by the demonstration that these assumptions are untenable. It must, however, be borne in mind that although this difference between the dermatitis of the two species has been demonstrated, the underlying dietary deficiency in pellagra may yet be identical with the deficiency which directly produces rat dermatitis.

Suggestive but inconclusive evidence on this point is afforded by the observations recorded under part 1 of the experimental section, which show that a daily dose of 1 gm. of either the white or the yellow maize meal employed is sufficient to cure rat dermatitis. As the white maize meal was taken from the stock used in preparing diets for patients in the hospital, this observation appears to suggest that the substance (vitamin B_6) which cures rat dermatitis is not identical with the pellagra-preventive factor of Goldberger. This conclusion was considered highly probable by Birch and György ('35) who found that 0.5 gm. of maize meal was the minimum daily dose needed to cure rat dermatitis.

Turning now to the clinical evidence, it is to be noted that the Standard Basic Diet no. 2 contains 143 gm. of maize (92 gm. of meal and 51 gm. of grits); i.e., 286 times the minimum dose for the cure of rat dermatitis. The approximate ratio of weights of the human being and rat is about 600:1, so that weight for weight the patients received in the basic diet about half as much vitamin B_6 as the rats were given. At this level of feeding no improvement at all was seen in their condition. The amount of maize eaten by these patients during the development of their pellagra is likely to have been greater than that contained in Standard Basic Diet no. 2. Moreover,

Goldberger and Wheeler ('20) produced human pellagra experimentally by feeding to their subjects a diet containing about 250 gm. of maize daily or 500 rat day-doses of vitamin B_6. Therefore it is clear that 1) vitamin B_6 is a different entity from the pellagra-preventive factor, or 2) vitamin B_6 and the pellagra-preventive factor are identical, but the human requirement of this factor relative to weight is much greater than the rat's, or 3) either vitamin B_6 or the pellagra-preventive factor is a complex of which the other is one component. The weight of evidence appears at present to be in favor of the first alternative.

The clinical observations with lactoflavin again are suggestive but not final. In case I the patient received 17 mg. of lactoflavin over a period of 8 days, an amount corresponding to a daily average of 300 oral day-doses for a rat. This dosage given subcutaneously was considered to be substantially similar, relative to weight, to the minimum needed by the rat, but no improvement resulted. Replacement of the lactoflavin by Valentine's Liver Extract led to improvement in 3 days. In case 2 the patient received 4 mg. daily of lactoflavin over a period of 5 days and showed no definite improvement. He subsequently improved rapidly when given the potent liver preparation, but left the hospital with one symptom persisting. In case 3 the patient deteriorated in condition while receiving lactoflavin and then suddenly became worse and died. Thus no one of the three cases was carried through the full routine of the test as described above, and the evidence afforded by them is not final. It is indefinite but suggests that lactoflavin has no pellagra-curative activity. A further study of lactoflavin will be made during the pellagra season of 1936.

SUMMARY

1. Rat dermatitis appears among rats on a B_6 deficient diet as readily in the dark as in the light, and is therefore not analogous to the dermatitis of human pellagra.

2. One gram of white or yellow maize meal contains at least one 'curative day-dose' of vitamin B_6, suggesting that vitamin B_6 is not identical with the pellagra-preventive factor.

3. Preliminary clinical trials with lactoflavin suggest that lactoflavin possesses no pellagra curative activity.

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THE EFFECT OF FEEDING EGG YOLK ON THE LIVER LIPIDS OF YOUNG RATS

RUTH OKEY AND EDITH YOKELA
Laboratory of Household Science, University of California, Berkeley

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That the ingestion of lecithin may partially or wholly prevent the development of fatty livers by rats fed diets high in fat has been indicated by the studies of Best and Hershey ('32). The same group of investigators have reported that choline is the component of the lecithin to which this action may be ascribed (Best and Huntsman, '32; Best and Ridout, '33). Blatherwick and his co-workers ('33) were, however, able to demonstrate no effect on the liver lipids of rats fed egg yolk and liver diets when lecithin was given.

Channon and Wilkinson ('35) and Aylward, Channon and Wilkinson ('35), since the present investigation was well under way, have presented figures which indicate that, while choline does affect the deposition of fat in the livers of rats on a high fat diet, it has a much less marked, if appreciable, influence on the deposition of cholesterol ester.

Best's et al. ('35) most recently published results are again not entirely in agreement with Channon's.

From the point of view of practical application in dietetics the need for further investigation of the effect of lecithin on reaction to high cholesterol diets seems self evident. Almost all cholesterol rich foods are at the same time excellent sources of lecithin—e.g., egg yolk, brains, liver. In our own laboratory at a comparatively low level of cholesterol intake, the effect of egg yolk feeding on the blood cholesterol of normal women was quite comparable to that observed when the same women took cholesterol with fat alone (Okey and Stewart, '33).

The increase in the incidence of arteriosclerosis and gall bladder disease, especially among diabetics, is hard to explain without taking into consideration the possible effect that the use of large numbers of eggs in the dietary may have. A recent reviewer (Brody, '35) comments, "only humans die in early life from coronary sclerosis, only humans suffer almost universally from atherosclerosis with advance of age, only humans consume regularly during adult life cholesterol rich foods."

Nevertheless in one of the recent textbooks in dietetics Newburgh and McKinnon ('34) propose no less than eight out of ten skeleton therapeutic diets which call for the use of from five to seven eggs per person per day. Certainly a sufficiently large number of people survive dietary regimes in which eggs are used habitually to suggest that it may be to the point to investigate the results of egg consumption when the other constituents of the dietary are carefully controlled. Our present study has therefore dealt with the effect of egg yolk feeding on the liver lipids of rats on diets which are, in so far as can be determined, adequate to meet all nutritional requirements.

EXPERIMENTAL

The diets were made up to contain egg yolk, EYC, egg yolk protein plus neutral fat plus cholesterol, ECC, and egg yolk protein plus fat alone, EYF. (For details of composition see table 1.) Individual cages with 'safety' food cups were used, and the respective diets given ad libitum.

At the close of the experimental period the rats were killed, the tissues ground, and the alcohol-ether extracts prepared and analyzed as described in a previous paper (Okey, Gillum and Yokela, '34). Cholesterol was in all cases determined both colorimetrically and by digitonid precipitation, but for the sake of consistency with the other lipid analyses all figures given are for data from the microxidation procedure. Individual data are given in table 2 and summarized in table 3.

It will be seen that the livers of the egg yolk-fed animals contained cholesterol ester in quantities which, while appreciably less than those observed in the animals fed cholesterol with fat, still averaged more than twelve times the concentration in the controls. These livers were whitish, and presented much the same appearance of gross engorgement with lipid presented by those of the cholesterol fed controls.

Cholesterol intake and cholesterol storage in the liver. The growth of cholesterol fed animals will be discussed in a separate paper. It is necessary to point out, however, that the

TABLE 1

Composition of experimental diets

	DIET[1] EYC PARTS	DIET ECC PARTS	DIET EYF PARTS
Commercially dried egg yolk[2]	25.3	...	...
Alcohol-ether extracted egg yolk	...	8.4	8.4
Extracted casein	11.6	11.6	11.6
Agar	4.0	4.0	4.0
Salt (Osborne-Mendel)	3.0	3.0	3.0
Starch	58.1	58.0	58.0
Crisco	...	15.0	15.0
Cholesterol	...	1.0	...
Total lipid	16.0	16.0	15.0
Total protein	17.5	17.7	17.7
Calories per gram; diet (excluding cholesterol)	4.4	4.5	4.5

[1] Vitamin supplements: Yeast, 0.5 gm. rat/day; CLO, 2 drops/rat/day.

[2] Composition: Protein (N $\times$ 6.25) 29.2 per cent, alcohol-ether ext. 63.8 per cent, lecithin 9.6 per cent, ash 3.3 per cent, cholesterol 3.9 per cent.

growth of both egg yolk-fed and cholesterol-fed controls compared very favorably with that of animals on a very good stock diet.

The experimental diets were planned to furnish as nearly an equal number of calories per gram food as possible. Nevertheless, the egg yolk-fed animals ate so much less per unit gain in weight that in the animals fed only 60 days the cholesterol intake of the cholesterol-fed control group (ECC) per 100 gm. body weight was about 1.4 times that of the group fed the egg yolk. The rats fed cholesterol as such show liver

TABLE 2

Liver lipids of rats

NO.	SEX	WEIGHT RAT GRAMS	WEIGHT LIVER GRAMS	MOISTURE, PER CENT	FATTY ACID	TOTAL CHOL.	ESTER CHOL.	FREE CHOL.	LECITHIN	LIVER CHOL. MG. RAT
					Per cent moist weight liver					
				Diet E.Y.C.—60 days						
59	♂	325	14.9	60.3	12.5	3.00	2.77	0.23	3.1	448
61	♂	253	8.8	62.7	8.8	2.45	2.15	0.30	2.9	215
68	♂	265	9.1	57.7	13.5	2.84	2.51	0.33	3.2	251
71	♂	289	10.3	64.1	7.8	2.23	1.95	0.28	2.8	230
P(3)[1]	♂	196	9.7	63.8	9.3	2.58	2.31	0.27	2.7	250
69	♀	188	7.2	60.3	12.6	4.05	3.76	0.29	2.7	293
70	♀	160	6.4	...	7.7	2.19	1.79	0.40	3.0	136
72	♀	176	5.8	61.4	11.5	4.10	3.73	0.37	2.7	232
73	♀	165	6.2	59.6	12.0	6.50	6.20	0.33	2.6	403
P(3)[1]	♀	172	6.1	62.4	11.7	3.86	3.52	0.34	2.5	237
				Diet E.Y.C.—120 days						
58	♂	316	9.2	68.0	5.6	1.13	0.89	0.24	2.5	104
60	♂	325	9.0	63.0	5.7	2.46	2.12	0.34	2.8	224
66	♂	356	10.9	60.9	5.7	5.33	4.97	0.38	2.6	580
67	♂	361	11.8	64.6	7.6	2.46	2.06	0.40	2.8	280
62	♀	225	7.6	56.2	16.7	3.50	3.26	0.24	2.3	266
63	♀	210	7.8	59.0	14.8	3.56	3.25	0.31	1.5	278
64	♀	238	8.5	55.0	20.5	5.12	4.32	0.38	2.2	436
65	♀	210	7.5	52.6	19.6	5.75	5.45	0.30	2.0	434
				Diet E.C.C.—60 days						
04	♂	213	11.4	48.4	25.6	7.15	6.92	0.23	2.8	810
07	♂	224	9.1	55.4	18.9	4.85	4.63	0.22	3.2	443
10	♂	251	11.9	56.1	17.8	4.50	4.30	0.20	3.0	534
12	♂	203	9.7	...	19.3	7.35	7.12	0.23	3.0	710
03	♀	148	8.2	57.8	17.9	3.46	3.10	0.36	2.1	620
06	♀	162	7.7	58.0	17.6	5.50	4.14	0.36	2.6	425
08	♀	145	8.2	51.9	20.1	8.20	7.82	0.38	1.8	677
09	♀	143	7.8	57.8	17.1	7.20	6.83	0.37	1.9	560
				Diet E.C.C.—120 days						
02	♂	350	14.6	61.0	10.9	4.30	4.15	0.15	2.5	627
05	♂	295	15.7	51.6	21.0	6.20	6.04	0.15	2.2	970
11	♀	225	8.9	64.8	11.5	2.60	2.50	0.10	2.7	260
13	♀	243	10.4	58.4	16.7	5.45	5.32	0.13	2.3	570
				Diet E.Y.F.—60 days						
14	♂	220	6.9	70.3	3.8	0.25	0.02	0.23	2.0	17
49	♂	203	6.8	63.3	4.9	0.29	0.06	0.23	1.9	20
52	♂	210	7.1	67.6	5.9	0.29	0.01	0.28	2.4	21
53	♂	207	7.0	70.0	4.5	0.29	0.01	0.28	2.5	20
P(6)[1]	♂	208	8.3	65.9	7.5	0.48	0.18	0.30	2.7	40
16	♀	190	6.9	67.1	8.1	0.27	0.03	0.24	2.3	18
17	♀	178	6.6	67.8	6.5	0.22	0.00	0.22	2.2	13
50	♀	154	5.1	67.7	7.2	0.24	0.00	0.25	2.5	13
51	♀	154	6.0	67.8	5.1	0.24	0.01	0.24	2.6	15
P(6)[1]	♀	152	8.0	66.1	7.4	0.30	0.01	0.29	2.0	20
				Diet E.Y.F.—120 days						
15	♂	285	8.8	65.4	7.9	0.33	0.13	0.20	2.6	29
55	♂	253	7.3	68.5	5.1	0.28	0.11	0.17	2.7	20
48	♀	190	5.6	73.4	7.3	0.29	0.04	0.25	2.3	16
54	♀	196	5.7	...	7.5	0.28	0.07	0.21	2.0	20

[1] Pooled samples from the number of rats indicated.

TABLE 3

Summary of data. Determinations for individual animals: 60- and 120-day groups taken together. Equal numbers of males and females in each group

	EYC	ECC	EYF	DIFFERENCES		
				EYC and ECC	EYC and EYF	ECC and EYF
Weight rat grams	254 ±11.8	217 ± 12.2	203 ±6.9	37 ± 17	51 ±13.7	14 ±14
Weight liver grams	8.8 ± 0.39	10.3 ± 0.51	6.6 ±0.20	1.5 ± 0.20	2.2 ± 0.44	3.7 ± 0.55
Total F.A. per cent moist weight	11.4 ± 0.81	17.9 ± 0.76	6.1 ±0.28	6.5 ± 1.11	5.2 ± 0.86	11.7 ± 0.8
Total chol. per cent moist weight	3.5 ± 0.25	5.6 ± 0.27	0.27±0.01	2.0 ± 0.37	3.26± 25	5.3 ± 0.27
Ester chol. per cent moist weight	3.2 ± 0.25	5.2 ± 0.30	0.05±2.0	2.0 ± 0.38	3.15± 25	5.2 ± 0.30
Free chol. per cent moist weight	0.32± 0.01	0.24± 0.02	0.23±0.01	0.08± 0.023	0.09± 0.014	0.01± 0.023
Lecithin per cent moist weight	2.62± 0.07	2.52± 08	2.33±0.10	1.10± 0.111	0.29± 0.16	0.19± 0.16
Total liver chol. mg./rat	301 ±17.7	601 ±125	18.6 ±0.86	300 ±126	282 ±17.7	582 ± 0.9

EYC = egg yolk diet.
ECC = extracted egg yolk with cholesterol and Crisco.
EYF = extracted egg yolk with Crisco only.

cholesterol and fatty acid values consistently about 1.5 to 2 times those of the egg yolk-fed animals. At the end of the period of rapid growth the differences in food intake were very much less marked, and this was also true of the differences in cholesterol ester concentration in the liver, the respective mean being, cholesterol-fed group 4.50 per cent and the egg yolk-fed group 3.29 per cent.

The livers of the egg yolk-fed and control animals did not continue to enlarge as they grew older, but the small group fed cholesterol show a rather great increase in size. This suggests that the continued enlargement of the liver with age accompanied by increased storage of cholesterol ester is the chief defense mechanism against cholesterol invasion of other tissues in the rat fed cholesterol without lecithin. The egg yolk-fed animals, on the other hand, had no larger livers at 120 than at 60 days, and only very little more liver cholesterol per rat. This must indicate that their cholesterol is either moved to other tissues and deposited or that it is destroyed.

Effect of fat intake and age of animals

Cholesterol feeding. In order to utilize egg yolk powder to furnish cholesterol at a 1 per cent level, the total lipid concentration of the diet other than cholesterol was automatically raised to 16 per cent. Comparison with our figures for control animals fed 5 and 10 per cent fat shows that this in itself apparently had no effect on the fatty acid concentration of the livers. That of the cholesterol-fed animals was approximately 2 per cent greater (mean 19.3 per cent) than the mean of our previous observations on animals fed 5 and 10 per cent fat in the diet. Cholesterol ester concentrations varied only within the limits to be expected to be accounted for by chance in the 60-day control groups, i.e., for animals 83 to 85 days old.

Both fatty acid and cholesterol were lowered in the livers of the animals fed cholesterol for 120 days and varied hardly at all in the fat fed controls.

Egg yolk feeding. In the case of the animals fed egg yolk, however, there seemed to be a tendency for the fatty acid

concentration of the livers of the males to decrease and of the females to increase with age.

The series fed egg yolk for 120 days was not large, but the fact that the average liver fatty acid concentration in the females fed egg yolk for 120 days was almost three times that of the males, and one and three-quarters that of the females fed only 60 days, is at least interesting. Why the fatty acid concentration in the livers of the males fed egg yolk should decrease with age while that of the females increases is hard to explain as is the reason for the very much lower concentration of cholesterol ester in the livers of the male egg yolk-fed rats of both age groups.

If we assume that the holding back of cholesterol as ester by the liver is a defense mechanism designed to prevent its delivery to and accumulation in the tissues in which its presence is most dangerous to life processes, such for instance as the intima of the coronary arteries, we may correlate this sex difference in the response of the liver to continued ingestion of egg yolk diets with the higher incidence of arteriosclerosis in men, and of diseases associated with hepatic function in fat and cholesterol metabolism in women, e.g., gall stones.

Certainly the differences between the cholesterol-fed controls and the egg yolk-fed rats indicate some sex variation in the part played by the lecithin. In view of Best's findings it is especially interesting that the egg yolk-fed females had higher liver fatty acid values than the animals of either sex fed cholesterol without lecithin. Likewise the egg yolk-fed animals of both sexes had slightly higher total liver cholesterols at 120 days than at 60 days, a tendency apparently more manifest even than that of animals fed cholesterol without lecithin to increase liver cholesterol concentration with age.

These findings have led us to undertake to study the effect on the distribution of cholesterol in the tissues of feeding cholesterol and egg yolk diets during the entire life span of the rat. Further discussion is postponed until this can be done.

SUMMARY AND CONCLUSIONS

Rats fed diets containing egg yolk to furnish 1 per cent cholesterol tend to develop fatty livers in spite of the 2.3 per cent phospholipid content of this diet. The accumulation of fat and cholesterol ester tends, in females fed for 120 days, to be even greater than in animals fed egg yolk protein and cholesterol at the same level with hydrogenated vegetable oil[1] to replace the egg yolk lipid and no lecithin. Males tend to store less liver fat and cholesterol on egg yolk than on cholesterol diets.

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[1] Crisco.

THE COMPARATIVE EFFECTS OF COD LIVER OIL, COD LIVER OIL CONCENTRATE, LARD AND COTTONSEED OIL IN A SYNTHETIC DIET ON THE DEVELOPMENT OF NUTRITIONAL MUSCULAR DYSTROPHY

LOUIS L. MADSEN[1]
Departments of Pathology and Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York City

TWO PLATES (EIGHT FIGURES)

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When some herbivorous animals, namely, guinea pigs and rabbits (Goettsch and Pappenheimer, '31; Woodward and McCay, '32), as well as goats and sheep (Madsen, McCay and Maynard, '33, '35) are fed certain synthetic and semi-purified rations containing the known dietary essentials, nutritional failure usually results. A prominent cause of death on such rations is the development of a progressive, highly specific degeneration of the skeletal muscles.

During experimental inquiry into the fundamental food requirements of Herbivora by means of the synthetic diet technic results were obtained by Madsen, McCay and Maynard ('33, '35) which indicated that addition of cod liver oil to the basal ration increased the rate of development and early severity of the muscle degeneration. The substitution of a cod liver oil concentrate in the synthetic ration greatly delayed the production of muscle lesions and allowed longer survival and more growth, but did not prevent the eventual development of muscle dystrophy. Moreover, the entire elimination of cod liver oil from the synthetic diet and the use of carotene and irradiated yeast as a source of the A and D vitamins did not prevent the development of the muscle degeneration. Muscle lesions were also produced in guinea

[1] National Research Council Fellow in the Biological Sciences, 1934–1935.

pigs fed a stock diet supplemented with cod liver oil, but the natural foods supplied considerable protection against the development of lesions. These results indicated that both cod liver oil and other factors of the synthetic diet affected the production of muscle dystrophy in the animals studied.

This report deals with experiments on the production of muscle dystrophy which were planned to define more clearly the effect of cod liver oil and a vitamin A-D concentrate of cod liver oil as supplements to rations containing only lard or cottonseed oil as the main source of fat. These comparisons were made principally with a synthetic diet and also with a modification of diet 11 used by Goettsch and Pappenheimer ('31). The effect of a diet of grains with and without a supplement of cod liver oil was also studied with guinea pigs.

The experimental animals were killed at intervals or were examined at death after being on the diets for varying lengths of time. The presence of muscle changes was determined, by gross and histopathological examination. In many cases oxygen consumption and creatine determinations[2] were made on the excised muscle tissue. The data obtained with guinea pigs and rabbits are considered together, since the experiments and results were largely comparable.

EXPERIMENTAL WORK

Methods. Young guinea pigs weighing from 202 to 380 gm. and rabbits weighing between 520 and 1440 gm. were changed gradually from a normal stock diet to a basal synthetic ration of the following general composition:

	Per cent
Purified cellulose (sylphrap)	20
Casein	15
Sucrose	10
Starch	43 (or less)
Fat (lard or cottonseed oil)	3 (or more)
Yeast	5
Mineral mixture (Hawk-Oser, '31)	4
	100

[2] I am indebted to Dr. Marianne Goettsch for the creatine determinations included in this report.

Seven groups of guinea pigs and six groups of rabbits were fed this basal ration with variables as to the source and amount of fat and the source of vitamins A and D. The diets used in this study are summarized in table 1.

The guinea pigs and rabbits of group A were fed the basal synthetic ration containing either lard or cottonseed oil supplemented with cod liver oil. In rations A-2, A-3 and A-4, where more than 3 per cent of either lard or cottonseed oil was included, the composition of the diet was adjusted by decreasing the percentage of starch. The cod liver oil fed to guinea pigs of groups A-1 and A-3 was emulsified in a daily allowance of 7 to 10 cc. of tomato juice containing 0.2 gm. dried yeast. The cod liver oil supplement for the rabbits of groups A-2 and A-4 was included in the ration on a percentage basis. No source of antiscorbutic vitamin was provided in the rabbit diets.

TABLE 1

Diets fed to guinea pigs and rabbits

GROUP	BASAL DIET	SOURCE OF FAT	SOURCE OF VITAMINS A AND D	EXPERIMENTAL ANIMALS	
				Guinea pigs	Rabbits
A-1	Synthetic	3% lard	0.5 gm. CLO daily	5	..
2	Synthetic	4% lard	2.0% CLO	..	8
3	Synthetic	3 and 6% CSO[1]	0.5 gm. CLO daily	10	..
4	Synthetic	4 and 8% CSO	2.0% CLO	..	6
B-1	Synthetic	6% lard	0.05% A-D concentrate[2]	..	2
2	Synthetic	3% CSO	8 mg. A-D concentrate in 0.5 gm. CSO daily	10	..
3	Synthetic	6%CSO	0.05 % A-D-concentrate	..	6
C-1	Synthetic	None added	0.05% A-D concentrate	5	3
2	Synthetic	4% Sap. CLO	0.05% A-D concentrate	5	..
3	Synthetic	4% Sap.CSO	0.05% A-D concentrate	5	..
D-1	(Modified diet 11)	3.2% lard	0.5 gm. CLO daily	5	..
2		3.2% lard	8 mg. A-D concentrate in 0.5 gm. CSO daily	5	..
E-1	Grains	....	....	10	..
2	Grains	....	0.5 gm. CLO daily	5	..
F-1	Normal	....	....	5	4

[1] Cottonseed oil fed as Wesson oil.

[2] The A-D concentrate of cod liver oil was kindly furnished by Dr. T. F. Zucker.

The synthetic diets fed to rabbits of group B were supplemented with 0.05 per cent of A-D concentrate and the lard (group B-1) and cottonseed oil (group B-3) were increased to 6 per cent to equalize the total fat content of the rations. The guinea pigs of group B-2 were supplied with 3 per cent of cottonseed oil in the basal ration and the A-D concentrate was fed in a solution of 0.5 gm. cottonseed oil daily which was emulsified with yeast and tomato juice as previously described.

Previous experiments by Madsen, McCay and Maynard ('35) have shown that guinea pigs fed a synthetic diet containing lard and the saponifiable fraction of cod liver oil plus carotene and irradiated yeast as the source of the vitamins A and D developed a severe muscular degeneration. This indicated that a ration containing cod liver oil or its saponifiable fraction resulted in the development of muscle changes of a similar character. However, later work with rabbits during the present study suggested that the fat component of the diet should be considered as a possible variable in the production of muscle lesions. This relation was studied further by feeding the synthetic diet without added fat as a control diet (group C-1) and with the addition of the saponifiable fraction of cod liver oil (group C-2) and of cottonseed oil (group C-3) to the basal synthetic diet. These rations were supplemented with the A-D concentrate.

Two groups of guinea pigs were used to compare the use of cod liver oil (group D-1) and the A-D concentrate in cottonseed oil solution (group D-2) as a supplement to diet 11 which has been shown by Goettsch and Pappenheimer ('31) to produce muscle dystrophy in rabbits and guinea pigs. Diet 11 was modified by reducing the lard and it contained the following ingredients:

Rolled oats	355	Lard	30
Wheat bran	180	NaCl	10
Skim-milk powder	275	$CaCO_3$	15
Casein	75		

Previous work in this laboratory by Goettsch and Pappenheimer (unpublished data) has shown that if rabbits are fed

a diet consisting of grains alone a type of degeneration of the skeletal muscles will develop. This grain mixture which consisted of oats 65, barley 85, wheat 45 and wheat bran 5 was finely ground and fed to guinea pigs without (group E-1) and with (group E-2) a supplement of cod liver oil.

Animals of group F-1 were normal controls and received a stock ration which consisted of grains as fed to group E with the addition of alfalfa hay and lettuce.

The pathology and histology of nutritional muscle dystrophy has been described by Goettsch and Pappenheimer ('31) and Madsen, McCay and Maynard ('35). Some advanced stages of muscle degeneration are shown in plate 1. A detailed histological examination of several muscles from each experimental animal is essential, since a single muscle may or may not prove to be a reliable index as to the extent of the muscle lesions. The characteristic symptoms of muscle involvement are not usually obvious in the initial stages of the disease, however, advanced changes can readily be detected.

Routine muscle samples for histological examination were taken from the quadriceps, gastrocnemius, semitendinosus, adductor of the thigh, triceps, biceps, pectoralis and heart muscle. Occasionally, samples were taken from the masseter, tongue, diaphram, intercostals, soleus, and eye muscles. For purposes of tabulation the extent of the muscle lesions are graded from slight one-plus degeneration to the more severe two-, three- and four-plus changes as shown in plate 2 of a previous publication (Madsen, McCay and Maynard, '35).

Various organ samples were also taken for histological study. The liver of the dystrophic guinea pig is often larger than normal. The kidneys of both rabbits and guinea pigs were occasionally mottled, pitted and scarred. Cases of the so-called spontaneous medial calcification of the aorta were seen rather frequently in rabbits. A terminal bronchopneumonia developed in some advanced cases. Skeletal muscle dystrophy frequently occurs in cases apparently free from complicating diseases which seems to emphasize that the disease is highly selective and specific of skeletal muscle.

Some of the guinea pigs were killed at approximately 10-day intervals for histological examination and for oxygen consumption and creatine determinations on the skeletal muscles. Other animals were allowed to continue for longer periods on their respective diets. At intervals, under ethyl ether anesthesia, pieces of adductor muscle of the thigh were excised from some of the surviving guinea pigs and rabbits for respiration studies and histological examination.

The oxygen consumption determinations were made by the use of Fenn volumeters after the method outlined by Victor ('34). The value recorded as the oxygen consumption represents the average cubic millimeters of oxygen consumed per gram of moist tissue per minute during the second hour of the experiment. The muscle tissue was cut with scissors into fine longitudinal strips and a weighed sample of 75 to 200 mg. was placed into Ringer's solution containing a phosphate buffer of pH 7.4. The respirometers were flushed out with pure oxygen and then immersed and shaken in a water bath at a temperature of $37.5 \pm 0.005°C$. The oxygen consumption was followed for varying lengths of time between 2 and 8 hours.

The presence of muscle degeneration can usually be detected histologically in the portion of muscle used in the respiration determination, but the extent of the muscle change may be somewhat confusing, since the sarcoplasm may be altered during the experiment. In the case of rabbits, where plenty of tissue is available, some of the original biopsy material was fixed in Zenker's solution immediately and the remaining portion was used for the oxygen consumption determination and then fixed for microscopic study. Both samples were embedded together for sectioning and then mounted and stained in the usual manner.

Creatine was determined colorimetrically in muscle tissue as described by Goettsch and Brown ('32), and the comparisons were made with the use of a photo-electric colorimeter.

Results. The incidence of muscular lesions in the animals fed the various experimental diets will be given first and will

be followed by a presentation of the results of the oxygen consumption and creatine determinations.

The data for the rabbits and guinea pigs fed the synthetic diet containing either lard or cottonseed oil and supplemented with cod liver oil are summarized in tables 2 and 3. The animals developed muscle dystrophy very rapidly when fed these diets while the guinea pigs on the normal diet, as usual, failed to develop muscular lesions as shown in table 2. Slightly greater growth and longer survival resulted in several cases when the animals were fed the synthetic diet containing cottonseed oil as the source of fat as compared to lard; however, muscle dystrophy occurred in the animals after about the same interval on both of these rations which were supplemented with cod liver oil as the source of vitamins A and D.

Degenerative changes in the heart muscle consisting of necrosis of muscle fibers, cellular infiltration, calcification and fibrosis were noted in rabbits nos. 3, 14, 5 and 2 (plate 2) and also in rabbits 13 and 15. These areas of degeneration were evident in the gross. In rabbit no. 2 the heart lesion was primarily a vascular one and consisted of intimal thickening and fragmentation of the inner elastic membrane of the arterial walls. There was some fibrosis present in the myocardium in the region of the altered vessels. Heart involvement is not a consistent finding in rabbits or guinea pigs with severe skeletal muscle degeneration.

A summary of the data obtained when the basal synthetic diet was supplemented with an A-D concentrate of cod liver oil instead of cod liver oil are given in table 4.

The two rabbits fed the synthetic diet in which lard provided the main source of fat (group B-1) developed advanced muscle lesions, but the data obtained with guinea pigs and rabbits fed the synthetic diet containing cottonseed oil as the source of fat and the A-D concentrate as the vitamin supplement (groups B-2 and B-3) show an interesting contrast to the results obtained with the previous rations. Only one guinea pig out of ten in group B-2, no. 191, developed muscle

TABLE 2

Summary of results with guinea pigs and rabbits fed a synthetic diet containing lard as the main source of fat and supplemented with cod liver oil as compared to guinea pigs on the normal diet

GROUP	ANIMAL NO.	DAYS ON DIET	BODY WEIGHT Initial	BODY WEIGHT Days to maximum	BODY WEIGHT Maximum	BODY WEIGHT Final	RESULT	SKELETAL MUSCLE DYSTROPHY	OXYGEN CONSUMPTION C.MM./GM./MIN.	MUSCLE CREATINE[1] MG./100 GM. Sample	1	2
			gm.		gm.	gm.						
A-1 (guinea pig)	165 ♀	12	256	10	284	266	Normal, killed	—	1.18	Mixed	520	518
	161 ♂	21	240	17	306	218	Died,	+ to ++		Mixed	342	404
	162 ♂	33	330	15	384	367	P+,[2] killed	— to ++	2.44	Mixed	422	383
	164 ♀	49	272	35	386	333	P+++, killed	++++	6.57	Triceps	127	
										Quadriceps	105	99
	163 ♂	56					Weak, biopsy	+++	2.02			
	163 ♂	58					Weak, biopsy	++++	2.05	Quadriceps	73	
	163 ♂	59	202	47	354	256	Died,	++++		Triceps	74	
A-2 (rabbit)	2 ♂	0					Normal, biopsy	—	0.57	Semitendinosus	287	
	2 ♂	14	1130	7	1230	970	P++, died	++++		Gluteus	387	344
										Soleus	219	
	14 ♀	0					Normal, biopsy	—	0.50			
	14 ♀	15					P—, biopsy	—	1.18			
	14 ♀	33					P±, biopsy	++	2.03	Red muscle	268	
	14 ♀	40	1300	17	1610	1270	P+++, killed	++++	2.98	White muscle	260	
	15 ♀	0					Normal, biopsy	—	1.65	Soleus	222	
	15 ♀	15					P—, biopsy	—	2.06	Gastrocnemius	343	
	15 ♀	26	1440	24	1540	1450	P+++, killed	+++	1.47	Adductor	225	445
	5 ♂	0					Normal, biopsy	—	1.63	Gastrocnemius	366	
	5 ♂	15					P—, biopsy	—	1.45	Quadriceps	399	
	5 ♂	17	1060	3	1100	900	P—, died	+++		Soleus	322	
	22 ♀	6	740	1	740	550	Moribund, killed	±		Semitendinosus	319	
	23 ♀	31	750	16	860	640	P++++, killed	++++				
	24 ♀	47	740	7	790	450	P+++, died	++++				
	25 ♂	45	950	26	1030	710	P+++, died	++++				
F-1 (guinea pig)	172 ♀	80	250	80	520		Normal, biopsy	—	0.54			
	173 ♀	47	220	47	496	496	Normal, killed	—	1.34	Mixed	416	478
	193 ♂	83					Normal, biopsy	—	1.24			
	193 ♂	208	219	194	920	910	Normal, killed	—	2.72	Mixed	372	
	194 ♂	36	216	36	417	417	Normal, killed	—	1.07	Mixed	399	413
	195 ♂	73					Normal, biopsy	—	0.54			
	195 ♂	209	186	209	800	800	Normal, killed	—	3.12	Mixed	381	

[1] Creatine determinations were made only on muscles at death of animal.
[2] Symptoms of paralysis the extent of which is indicated by plus signs.

TABLE 3

Summary of results with guinea pigs and rabbits fed a synthetic diet containing cottonseed oil as the main source of fat and supplemented with cod liver oil

GROUP	ANIMAL NO.	DAYS ON DIET	BODY WEIGHT: Initial	BODY WEIGHT: Days to maximum	BODY WEIGHT: Maximum	BODY WEIGHT: Final	RESULT	SKELETAL MUSCLE DYSTROPHY	OXYGEN CONSUMPTION C.MM./GM./MIN.	MUSCLE CREATINE[1] MG./100 GM.: Sample	1	2
			gm.		*gm.*	*gm.*						
A-3 (guinea pig) (3% CSO)	169 ♂	14	264	5	272	268	Normal, killed	—	1.02	Mixed	540	
	168 ♂	22	266	13	318	287	Normal, killed	—	1.53	Mixed	475	441
	167 ♂	35	314	35	375	375	Normal, killed	±	1.62	Mixed	412	408
	170 ♀	57					Normal, biopsy	±	0.62			
	170 ♀	99					P±,[2] biopsy	+++	5.93			
	170 ♀	150					P+, biopsy	++	3.34			
	170 ♀	175	283	163	540	460	P++, killed	++++	2.48	Mixed	131	127
	166 ♀	56					P—, biopsy	±	1.75			
	166 ♀	82					P±, biopsy	+	1.86			
	166 ♀	153					P+, biopsy	±	1.49	Red muscle	296	
	166 ♀	191	280	191	520	520	P+, killed	+ to ++++	3.36	White muscle	212	
A-3 (guinea pig) (6% CSO)	206 ♂	35	272	29	400	339	P++++, killed	++++				
	207 ♂	55	256	53	318	314	P+++, killed	++++				
	208 ♂	38	326	11	404	329	Moribund, killed	++++				
	209 ♀	45	312	6	324	205	P++, killed	++++				
	210 ♀	46	348	34	360	296	P+, killed	++++				
A-4 (rabbit) (4% CSO)	19 ♂	0					Normal, biopsy	—	1.31			
	19 ♂	17					P—, biopsy	—	1.47	Red muscle	284	
	19 ♂	24	950	14	1010	830	P+, killed	++++	2.96	White muscle	310	
	3 ♂	0					Normal, biopsy	—	1.0			
	3 ♂	17					P—, biopsy	—	0.88			
	3 ♂	33					P++, biopsy	++	2.13	Red muscle	305	
	3 ♂	35	1220	27	1500	1050	P++, killed	++++	2.41	White muscle	331	
	13 ♀	0					Normal, biopsy	—	0.50			
	13 ♀	17					P—, biopsy	—	2.38			
	13 ♀	33					P—, biopsy	++	1.95			
	13 ♀	54					P+, biopsy	+++	3.84	Red muscle	154	
	13 ♀	75	1370	57	1520	1225	P++++, killed	++++	4.55	White muscle	136	
	18 ♂	0					Normal, biopsy	—	0.71			
	18 ♂	16					P—, biopsy	±	1.47			
	18 ♂	53					P+, biopsy	+	1.91			
	18 ♂	105	1135	89	1910	1330	P++++, killed	++ to ++++	2.74	Mixed	306	
A-4 (rabbit) (8% CSO)	26 ♀	48	670	23	890	570	P++++, killed	++++				
	27 ♀	58	950	33	1200	1190	P+, killed	++++				

[1] Creatine determinations were made only on muscles at death of animal.
[2] Symptoms of paralysis the extent of which is indicated by plus signs.

TABLE 4

Summary of results with guinea pigs and rabbits fed the synthetic diet containing lard or cottonseed oil supplemented with an A-D concentrate

GROUP	ANIMAL NO.	DAYS ON DIET	BODY WEIGHT Initial	BODY WEIGHT Days to maximum	BODY WEIGHT Maximum	BODY WEIGHT Final	RESULT	SKELETAL MUSCLE DYSTROPHY	OXYGEN CONSUMPTION C.MM./GM./MIN.	MUSCLE CREATINE[1] MG./100 GM. Sample	1	2
			gm.		gm.	gm.						
B-1 (rabbit)	16 ♂	0					Normal, biopsy	—	0.64			
	16 ♂	15					P—,[2] biopsy	+	1.04			
	16 ♂	38	1340	26	1360	1010	P++++ killed	++++	4.04			
	20 ♂	0					Normal, biopsy	—	1.40			
	20 ♂	17					P—, biopsy	+	1.54	Red muscle	220	
	20 ♂	47	1015	28	1270	1050	P++++ killed	++++	4.05	White muscle	201	
B-2 (guinea pig)	171 ♂	14	213	14	234	234	Normal, killed	—	1.46	Mixed	413	444
	192 ♂	25	268	12	312	200	Died, diarrhea	—		Mixed	463	455
	174 ♀	36	251	32	360	339	Normal, killed	—	1.02	Mixed	330	359
	191 ♂	57					Normal, biopsy	—	0.48			
	191 ♂	99					P—, biopsy	±	2.39			
	191 ♂	149					P—, biopsy	±	3.28			
	191 ♂	173	250	165	620	500	Moribund, killed	+		Mixed	468	
	176 ♀	56					Normal, biopsy	—	0.54			
	176 ♀	81					Normal, biopsy	—	1.17			
	176 ♀	152					Normal, biopsy	—	1.29	Red muscle	377	
	176 ♀	187	254	187	550	550	Normal, killed	—	2.89	White muscle	449	
	211 ♂	49	356	1	356	238	Moribund, killed	—				
	212 ♀	62	240	60	434	434	Normal, killed	—				
	213 ♂	5	296	1	304	254	Moribund, killed	—				
	214 ♀	57	294	18	335	324	Normal, killed	—				
	215 ♂	62	370	61	446	446	Normal, killed	—				
B-3 (rabbit)	17 ♂	0					Normal, biopsy	—	0.98			
	17 ♂	15					Normal, biopsy	—	1.54			
	17 ♂	32	1300	20	1580	1120	P—, died	—				
	7 ♂	0					Normal, biopsy	—	1.12			
	7 ♂	17					Normal, biopsy	—	0.68			
	7 ♂	47					Normal, biopsy	—	1.61	Red muscle	410	
	7 ♂	57	1000	46	1200	970	P—, died	—		White muscle	616	
	28 ♀	50	720	48	1320	1040	P—, died	+				
	29 ♀	51	520	48	830	800	P—, died	±				
	30 ♀	61	880	59	1200	1200	Normal, killed	+				
	31 ♀	62	700	45	1200	1080	Normal, killed	+				

[1] Creatine determinations were made only on muscles at death of animal.
[2] Symptoms of paralysis the extent of which is indicated by plus signs.

lesions and these consisted of scattered, atypical, necrotic fibers. This animal was killed when moribund, and at autopsy was found to have an extensive necrotizing bronchopneumonia. The creatine content of its muscle was within the normal range; however, the oxygen consumption was higher than normal on a previous biopsy. Four of the six rabbits in group B-3 died after an acute attack of diarrhea, the other two rabbits were killed after being on the diet for about 2 months. No gross symptoms of paralysis developed in these animals. At autopsy the muscles of these rabbits were apparently normal in color and consistency; however, on microscopical examination three of the rabbits were found to have several scattered necrotic muscle fibers of recent development. The growth and condition of the muscles of these animals was in marked contrast to the pale, streaked, atrophic and markedly degenerate muscles of the rabbits which were fed the basal synthetic diet supplemented with cod liver oil and had died of muscle dystrophy.

Three out of five of the guinea pigs and all of the rabbits fed the basal synthetic diet plus A-D concentrate without added fat developed characteristic muscle lesions as seen in table 5 (group C-1). However, the survival and growth of these animals was improved as compared to those fed the lard-cod liver oil rations in group A. In both groups of guinea pigs (C-2, C-3) in which the saponifiable fraction of cod liver oil and of cottonseed oil furnished the source of fatty acids a degeneration of their skeletal muscles also resulted.

As seen in table 6 all of the guinea pigs of group D-1 which received the supplement of cod liver oil, except the animal that was killed after 11 days on the ration developed some degree of muscle degeneration. In contrast only one of the five guinea pigs of group D-2 which was given a supplement of cod liver oil concentrate in cottonseed oil solution developed slight muscle changes during the course of the experiment.

The results obtained with guinea pigs fed the grain diet with and without the cod liver oil supplement are also given

in table 6. Guinea pigs nos. 186 to 190, inclusive, were started on experiment in October, 1934, while animals nos. 196 to 205 were placed on experiment in July, 1935. None of the first series of animals on the grain diet developed muscle dystrophy. However, two of the animals developed a type of flaccid paresis which was different from the stiffness or paralysis accompanying advanced muscular dystrophy. Several of the

TABLE 5

Summary of results with guinea pigs fed the synthetic diet without added fat, and the synthetic diet containing the saponifiable fractions of cod liver oil and cottonseed oil supplemented with an A-D concentrate

GROUP	ANIMAL NO.	DAYS ON DIET	BODY WEIGHT				RESULT		SKELETAL MUSCLE DYSTROPHY
			Initial	Days to maximum	Maximum	Final			
			gm.		gm.	gm.			
C-1 (guinea pig)	226 ♂	67	354	56	492	490	P±,	killed	+ to ++++
	227 ♂	66	300	56	384	352	P—,	killed	—
	228 ♂	67	316	40	444	410	P—,	killed	—
	229 ♂	61	358	56	452	411	P—,	killed	+
	230 ♂	43	330	35	400	355	P—,	died	++
C-1 (rabbit)	32 ♀	56	750	29	1050	980	P±,	killed	+++
	33 ♀	61	720	61	1050	1050	P±,	killed	++ to ++++
	34 ♀	62	770	59	1240	1210	P±,	killed	++ to ++++
C-2 (guinea pig)	216 ♀	62	300	62	454	454	P—,	killed	+
	217 ♂	47	380	41	410	336	P++++,	killed	++++
	218 ♀	55	296	50	364	302	P++++,	killed	++++
	219 ♀	63	336	43	433	420	P—,	killed	+
	220 ♂	56	312	41	464	409	P++++,	killed	++++
C-3 (guinea pig)	221 ♂	61	308	15	385	290	P++,	killed	++++
	222 ♀	38	289	25	297	214	P++,	died	++ to +++
	223 ♂	63	284	34	374	296	P—,	killed	+ to ++
	224 ♂	53	308	34	438	311	P+++,	died	++++
	225 ♂	57	314	34	399	349	P—,	killed	++

animals developed generalized edema and ascites which may have been due to the so-called nutritional edema resulting from the low intake of vegetable proteins.

Two of the animals fed the grain ration in the second series showed characteristic muscle changes. These results are confusing; however, when the data for group E-2 are considered it seems apparent that the addition of cod liver oil to the grain diet did increase the rate of development and severity of the

TABLE 6

Summary of results on guinea pigs fed modified diet 11 supplemented with cod liver oil and A-D concentrate and animals fed grains and grains plus cod liver oil

GROUP	ANIMAL NO.	DAYS ON DIET	BODY WEIGHT				RESULT	MUSCLE DYSTROPHY	OXYGEN CONSUMPTION C.MM./GM./MIN.	MUSCLE CREATINE[1] MG./100 GM.		
			Initial	Days on maximum	Maximum	Final				Sample	1	2
			gm.		*gm.*	*gm.*						
D-1	179 ♂	11	222	11	245	245	Killed, normal	—	1.67	Mixed	478	497
	182 ♀	34	238	12	304	283	Killed, weak	+ to ++	3.90	Mixed	320	370
	178 ♂	42	275	42	355	355	Killed, diarrhea	++	1.80	Mixed	349	335
	175 ♀	59	218	42	355	284	Killed, P—[2]	++++	3.38	Gastrocnemious	197	262
	177 ♂	78					Biopsy	+	2.35			
	177 ♂	112					Biopsy	±	0.57			
	177 ♂	150					Biopsy	±	2.83			
	177 ♂	206	266	206	750	750	Killed	+	2.81	Mixed	358	
D-2	185 ♀	12	272	12	299	299	Killed, normal	—	1.11	Mixed	492	512
	183 ♀	23	204	10	249	200	Died, diarrhea	—		Quadriceps	375	
	181 ♂	28	261	22	359	288	Killed, diarrhea	—	1.39	Mixed	437	416
	180 ♂	80					Biopsy	—	0.43			
	180 ♂	112					Biopsy	—	0.65			
	180 ♂	148					Biopsy	—	0.61			
	180 ♂	199	258	194	920	920	Killed, normal	—	2.05	Mixed	448	
	184 ♀	83					Biopsy	—	0.91			
	184 ♀	112					Biopsy	—	1.04			
	184 ♀	148					Biopsy	—	1.18			
	184 ♀	199	286	194	800	760	Normal, killed	+	2.75	Mixed	490	
E-1 grains	187 ♀	7	232	1	245	235	Normal, killed	—	0.96	Gluteus	469	
	189 ♂	21	223	12	258	216	Normal, killed	—	1.35	Mixed	498	528
	188 ♀	28	220	22	280	253	Killed, paresis	—	1.37	Mixed	423	415
	190 ♂	31	232	28	298	276	Died, paresis	—		Mixed	368	388
	186 ♂	50	216	7	222	200	Emaciated, killed	—	1.47	Triceps	486	
	196 ♀	52	315	34	326	214	P—, died	++				
	197 ♀	40	216	13	240	194	P-moribund, killed	—				
	198 ♂	54	234	13	246	205	P—, died	—				
	199 ♂	51	232	45	273	210	P-moribund, killed	++++				
	200 ♂	51	272	38	300	265	P-moribund, killed	—				
E-2 grains CLO	201 ♀	39	230	20	285	202	P+++, died	++++				
	202 ♂	47	210	38	236	190	P++, died	++++				
	203 ♂	23	272	22	292	282	P—, died	++++				
	204 ♂	47	232	36	234	180	P—, died	++++				
	205 ♀	41	240	29	288	245	P++moribund, killed	++++				

[1] Creatine determinations were made only on muscles at death of animal.
[2] Symptoms of paralysis the extent of which is indicated by plus signs.

muscle lesions during the experimental period. Growth and survival were very poor on both rations as would be expected from its numerous deficiencies. A daily supplement of 7 to 10 cc. of tomato juice furnished the antiscorbutic vitamin and the cod liver oil was fed emulsified in the tomato juice-yeast mixture.

The individual values obtained for oxygen consumption and creatine content of the excised dystrophic and normal skeletal muscles which are given in tables 2, 3, 4 and 6 are summarized in table 7.

TABLE 7

Signifiance of relations between the extent of degeneration, oxygen consumption and creatine content of the excised muscle tissue

OBSERVATION	ANIMAL	EXTENT OF DEGENERATION	NUMBER OF OBSERVATIONS	MEAN: Value	MEAN: Standard deviation	COEFFICIENT OF VARIABILITY
Oxygen consumption of muscles	Guinea pig	—	31	*c.mm./gm./min.* 1.27 ± 0.08	0.67	52
		±, +, ++	16	2.24 ± 0.16	0.93	42
		+++, ++++	7	3.68 ± 0.47	1.85	50
	Rabbit	—	21	1.20 ± 0.08	0.52	43
		±, +, ++	7	1.72 ± 0.10	0.39	23
		+++, ++++	9	3.23 ± 0.22	0.97	30
Creatine content of muscles	Guinea pig (mixed sample)	—	35	*mg./100 gm.* 442 ± 9.4	83	19
		±, +	5	427 ± 15.8	52	12
		++	8	366 ± 8.5	36	10
		+++, ++++	11	155 ± 15.4	76	49
	Rabbit (white muscle)	++++	7	248 ± 13.5	53	21
	(red muscle)	Same animals	7	281 ± 22.4	88	31

The oxygen consumption of the dystrophic guinea pig muscle is apparently increased as compared to the average value of 1.27 ± 0.08 c.mm. for the thirty-one determinations on animals maintained on several diets whose muscles failed to show histological evidence of degeneration. The mean value of 2.24 ± 0.16 c.mm. for muscles in the initial stages of degeneration can only be considered as suggestive of an increase, but the mean value of 3.68 ± 0.47 c.mm. for the +++ and ++++ dystrophic muscles appears significantly higher than normal in spite of the fewer determinations and high variability of the data.

Victor ('34) found an increase in the oxygen consumption of dystrophic rabbit muscle and additional data are provided in the present experiments. The average normal oxygen consumption of the excised rabbit muscle in this series was 1.2 ± 0.08 c.mm. as compared to 1.4 c.mm. as obtained by Victor. The mean value of 3.23 ± 0.22 c.mm. for the +++ and ++++ degenerated muscles suggests a significant increase in oxygen consumption with the degeneration, but because of the variability of the data and relatively few determinations the exact magnitude of the increase and its relation to the specific stage of degeneration is not clear. The method of grouping the oxygen consumption values on the basis of + to ++++ degeneration is probably not satisfactory since as yet this method does not take into consideration the type or stage but rather the amount of degeneration as pointed out by Goettsch and Brown ('32).

The rate at which oxygen is consumed by the degenerating muscle usually remains consistently high from the beginning of the experiment and falls only slightly with time, while that of the normal muscle falls rapidly during the first few minutes in the respirometer and then reaches a base level which is fairly constant. The biopsy data on guinea pigs no. 170, table 3, suggests that the oxygen consumption may increase to a maximum, and then decline even though the degeneration and paralysis continues. This may explain the relatively small increase obtained in such cases as guinea pig no. 163 of table 2. The oxygen consumption values on the samples of muscle obtained at biopsy for several other animals show an increase with increasing degeneration. The record of rabbit no. 13 in table 3 and rabbits nos. 16 and 20 in table 4 are of special interest in this regard. The data for other animals show a less marked but often a definite increase with degeneration, while for some animals the result was variable. In contrast to the apparent increase in respiration of dystrophic rabbit and guinea pig muscle Knowlton and Hines ('34) report that there is no change from normal in the oxygen consumption of atrophic gastrocnemii of rats produced by sciatic nerve section.

The data on muscle creatine show that muscle dystrophy in the guinea pig as well as in the rabbit (Goettsch and Brown, '32; Woodward, '32) is accompanied by a loss of creatine from the skeletal muscles which is roughly proportional to the extent of degeneration.

The so-called red muscles, represented for example by the semitendinosus and soleus in the rabbit are normally lower in creatine content than the white muscles, such as the adductors of the thigh, gluteus and quadriceps. Goettsch and Brown ('32) found an average of 314 $\pm$ 7.1 mg. creatine for the red and 463 $\pm$ 5.1 mg. creatine for the white muscles in rabbits. These normal data are used for comparison with the creatine content of muscles from rabbits with ++++ degeneration in the present series. Seven animals had an average creatine of 248 $\pm$ 13.5 mg. for the red and 281 $\pm$ 22.4 mg. creatine for the white muscle samples. This shows a marked decrease from normal and a relatively larger reduction of creatine in the white muscles as compared to the red in the same animal. A basis for this difference in reduction of creatine between red and white muscles is evident on histological examination since the red muscles usually maintain their integrity longer and are involved to a lesser extent in the degenerative process than the white muscles even in advanced stages of dystrophy.

DISCUSSION

The data presented in this paper (groups A-1, A-2) confirm the previous studies of Madsen, McCay and Maynard ('33, '35) in showing that when guinea pigs and rabbits are fed a synthetic diet containing lard as the principal source of fat and cod liver oil as a source of vitamins a severe degeneration of the skeletal muscles results. They also show that the substitution of cottonseed oil for the lard of this diet has probably no influence on the occurrence or severity of the dystrophy, although it may cause a slight improvement as regards growth and survival (groups A-3, A-4). However, the data obtained with this synthetic diet when the cod liver oil was

replaced by a concentrate of it fed in cottonseed oil solution suggest a protective effect of the cottonseed oil against the muscle degeneration (groups B-2, B-3). In the previous studies such a replacement, in which another A-D concentrate was fed in a cottonseed oil solution, markedly delayed the onset of the muscle lesions; but in the present experiment replacement by a concentrate without oil addition did not cause this favorable effect (group B-1). This suggests that the lard also may influence the muscle dystrophy or that the cottonseed oil was definitely protective. The elimination of lard from the diet, however, without the addition of cottonseed oil had little effect in reducing the occurrence of the lesions (group C-1). Thus it is indicated that animal fat per se is not a primary harmful factor. It is evident, however, that the protective effect of cottonseed oil is not effective where cod liver oil is fed instead of the concentrate at the levels used in this experiment (groups A-1 to A-4).

In the previous experiments (Madsen, McCay and Maynard, '35) a synthetic diet containing lard as the source of fat and supplemented with the saponifiable fraction of cod liver oil proved to favor the development of muscle lesions more than when the non-saponifiable portion was added to the diet. Further data show that the synthetic diet without added fat other than the saponifiable fraction of cod liver oil (group C-2) or of cottonseed oil (group C-3) with the concentrate as the source of vitamins produces severe muscle lesions. These experiments furnished little additional information except that no protective effect was obtained from this fraction of cottonseed oil, in contrast to the results with the oil as a whole (groups B-2, B-3).

The comparison with the modified diet 11 of Goettsch and Pappenheimer (groups D-1, D-2) resulted in lesions in all of the guinea pigs that were on the experiment longer than 11 days where cod liver oil was fed and only in a slight degree in one case when the ration was supplemented with a cottonseed oil solution of the concentrate. These results are noteworthy, as they are probably related to those obtained with the synthetic diets previously discussed.

The significant feature of the experiments (groups E-1, E-2) in which the grain diet was fed, was the development of lesions in all cases where cod liver oil was added and in two cases out of ten where the oil was omitted. This shows, in confirmation of the previous studies (Madsen, McCay and Maynard, '35) that cod liver oil is effective in the development of muscle degeneration with diets of natural foods as well as with the synthetic diets, and it is further demonstrated that cod liver oil is not the only factor concerned in this dietary disease. The diet of grains is obviously incomplete in several respects and among its deficiencies other contributory factors may be accounted for. No case of muscle dystrophy has been seen as yet in the control animals (group F-1) fed a stock ration consisting of grains as fed to group E and receiving in addition an abundance of alfalfa and lettuce. The effect of cod liver oil addition to this ration was not studied.

The data obtained on the oxygen consumption of excised dystrophic rabbit and guinea pig muscle are variable and limited but for the most part consistent in showing an increase as compared to normal values. Further work is necessary on this problem to determine the cause of this altered respiration, its relation to the specific stages of degeneration, and its relation to the respiration of other tissues as well as to the metabolism of the animal during life. The lowered creatine content of the degenerating skeletal muscles, the tendency to an increased oxygen consumption and similar histological picture of muscle lesions in rabbits and guinea pigs on the synthetic rations and the modified diet 11 of Goettsch and Pappenheimer, suggests that the disease is the same in both species.

It is recognized that the present experiments still leave much to be explained as to the causes of the dystrophy which is produced in Herbivora by dietary means. It is hoped in further studies to give attention to other components of the diet in addition to continuing studies on the fat interrelationships which have been the primary concern of the present experiment.

SUMMARY

A study is reported of the skeletal muscle dystrophy which occurs in guinea pigs and rabbits fed synthetic diets containing cod liver oil or its concentrate and with lard or cottonseed oil as the chief source of fat. The basal synthetic diet alone without added fat other than the non-saponifiable fraction of cod liver oil as a source of vitamins produced dystrophy to nearly the same extent as the same diet which included 6 per cent of lard; but the use of cottonseed oil in place of the lard resulted in a high degree of protection against the development of muscle lesions. No such protective effect resulted when cod liver oil was fed as the source of vitamins in place of its non-saponifiable fraction.

Experiments with restricted natural food diets demonstrated these relations further and furnished additional evidence that the fat relationships studied are probably not the only factors in the production of muscle lesions. Nutritional muscular dystrophy in the rabbit and guinea pig is similar histopathologically and is characterized by a lowered creatine content and a tendency to an increased oxygen consumption of the excised muscle tissue.

The cooperation and facilities provided by Dr. A. M. Pappenheimer, Dr. H. T. Clarke, Dr. M. Goettsch, Dr. J. Victor and Mrs. Anna Hart as well as the support of the National Research Council in the Biological Sciences which made this study possible are gratefully acknowledged.

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PLATE 1

EXPLANATION OF FIGURES

1 Rabbit 2. Group A-2. Fourteen days on diet. Section of masseter muscle showing hyaline degeneration of muscle fibers, multiplication of muscle nuclei, infiltration of cells and beginning calcification of necrotic fibers.

2 Rabbit 20. Group B-1. Forty-seven days on diet. Section of gastrocnemius muscle. Note almost complete disappearance of muscle fibers and the unusual picture of a multinucleated atrophic muscle fiber near the bottom of the figure as compared to the nearly normal fiber at the top.

3 Guinea pig 202. Group E-2. Forty-seven days on diet. Section of adductor muscle of the thigh showing massive necrosis of muscle fibers.

4 Guinea pig 170. Group A-3. One hundred and seventy-five days on diet. Cross section of quadriceps muscle showing scattered muscle fibers with replacement of previously degenerated muscle with adipose tissue.

PLATE 1

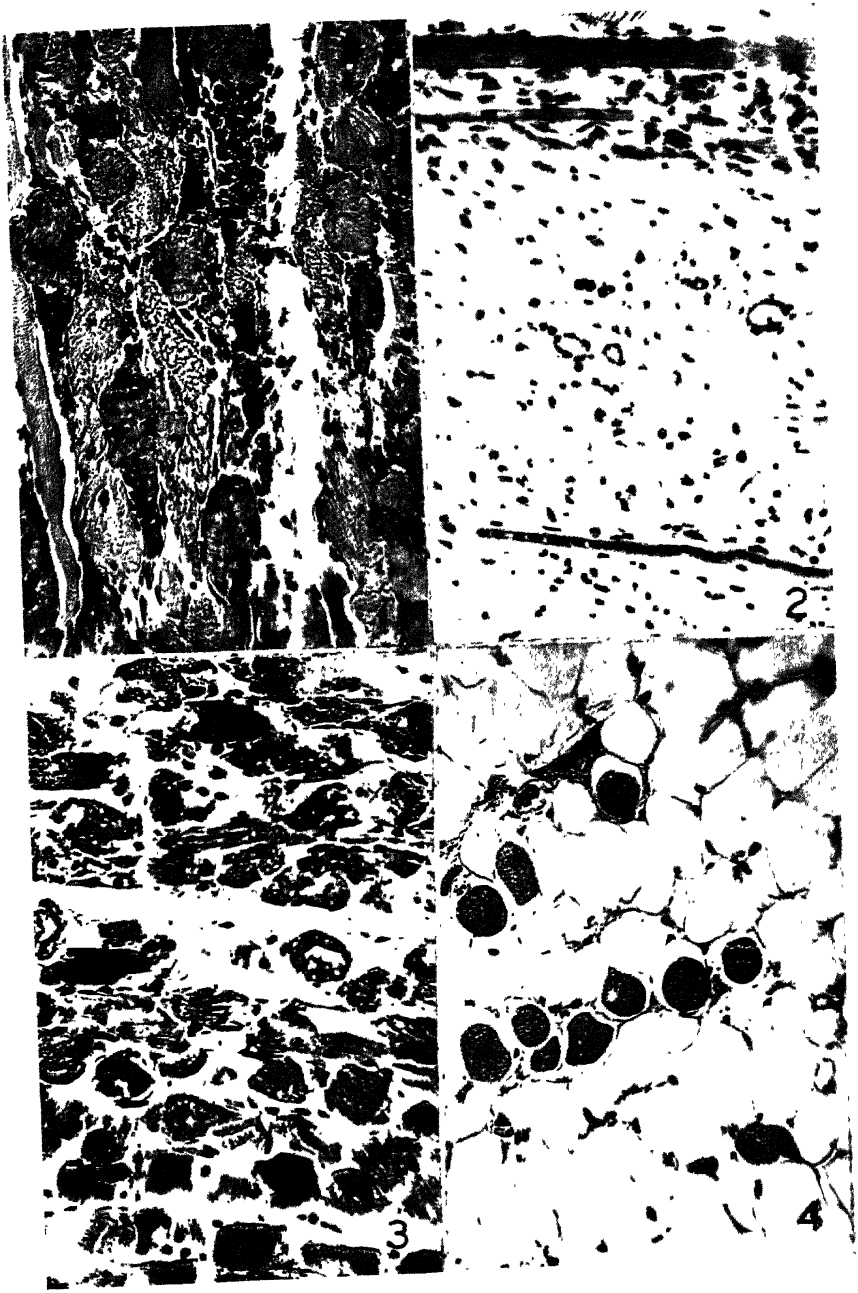


PLATE 2

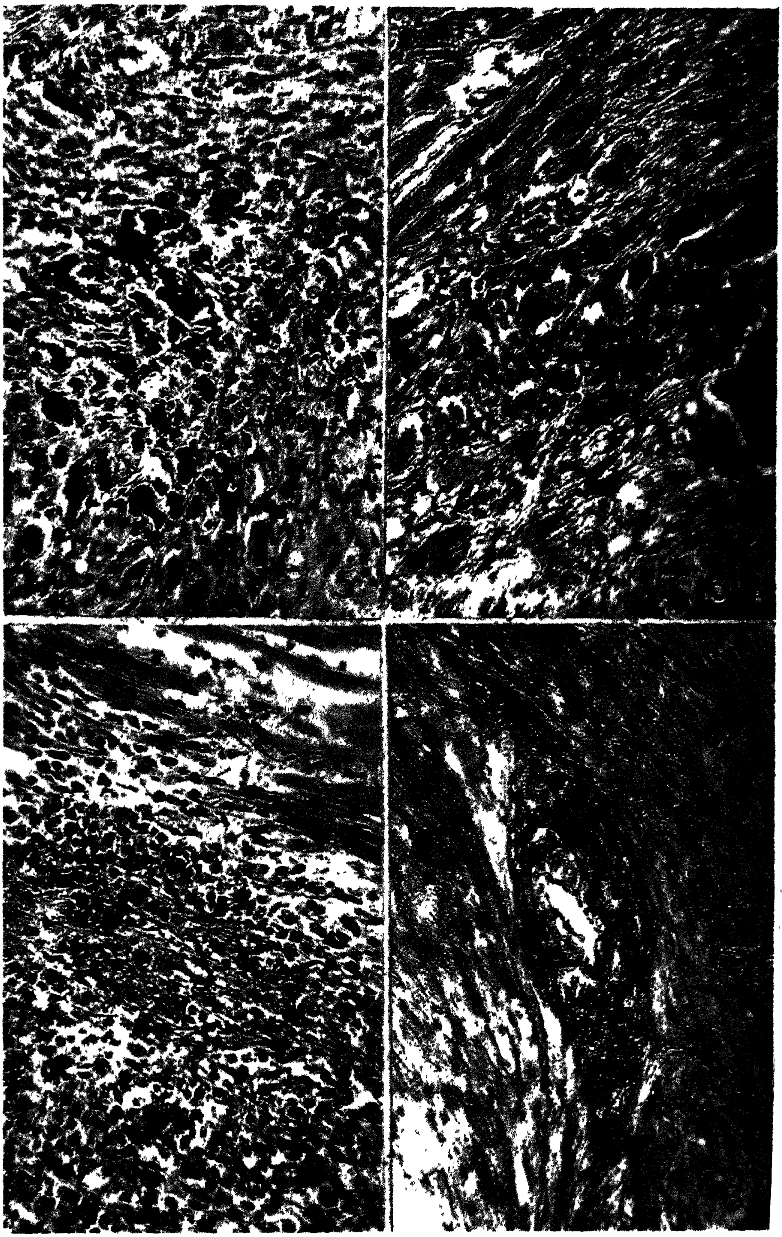
EXPLANATION OF FIGURES

5 Rabbit 3. Group A-4. Thirty-five days on diet. Section of cardiac muscle with atrophy and calcification of necrotic muscle fibers.

6 Rabbit 14. Group A-2. Forty days on diet. Section of cardiac muscle showing calcifications of necrotic muscle fibers, multiplication of muscle nuclei with some formation of multinucleated plasmatic masses.

7 Rabbit 5. Group A-2. Seventeen days on diet. Section of cardiac muscle. Degeneration of muscle with proliferation of cells about densely eosin staining fibers.

8 Rabbit 2. Group A-2. Fourteen days on diet. Small artery in wall of left ventricle showing intimal thickening and fragmentation of the inner elastic membrane.



THE USE OF 3-DAY PERIODS IN HUMAN METABOLISM STUDIES[1]

CALCIUM AND PHOSPHORUS

S. IDELL PYLE AND C. ELTON HUFF
WITH THE ASSISTANCE OF RAYMOND DAVIS
Samuel S. Fels Research Foundation, Antioch College, Yellow Springs, Ohio

TWO FIGURES

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A number of studies of mineral metabolism during pregnancy have been carried out in this country and abroad. Macy et al. ('30), Coons et al. ('34) and Donelson ('31) have developed an excellent mechanical technic for making such studies in the home, and after extensive experimentation have selectd a 4- to 6-day balance period as representative and adequate for studying mineral retentions. They recommend, too, that continuous balance periods where food ingestion varies from day to day be grouped into these shorter segments in order to bring out both the individual's variability and her average physiological tendencies throughout the prenatal period.

In attempting to adapt their methods to our studies of prenatal metabolism while women were living under normal home conditions, we considered the possibility of using a shorter balance period than those recommended by them. Duties in the home are multiple and a long period is often such a burden to the mother that the benefits do not seem to

[1] This is a report of a cooperative study carried out under the auspices of the Samuel S. Fels Fund for Research in Prenatal and Postnatal Environment under the direction of L. W. Sontag. The authors wish to express their appreciation to the persons who served as the subjects of this study.

warrant the time invested. We finally selected as a basis for experimentation a 24-hour period preceded by 2 consecutive days of preparation during which the food eaten was as identical qualitatively and quantitatively to the third, or collecting, day as the subject could make it within the limits of her appetite. Emphasis was put upon the need for similarity in preparing each food item eaten during the 3 days. This, of course, instituted a procedure unlike the ordinary habits of an adult since normally very few people eat exactly the same food prepared in exactly the same way for even 2 successive days. However, the need for approaching physiological stability seemed important if one were trying to get an idea of the metabolic state during pregnancy through intermittent sampling.

The object of this paper, then, is to present data gathered from the experiments so conducted and to compare them with the work done by Macy et al. ('30) and Coons et al. ('34) on the averaged 4- to 6-day periods in order to arrive at some conclusion as to variability, and to test the validity of 24-hour sampling periods which have been preceded by 2 days of preparation. Calcium and phosphorus metabolism have been considered first.

SUBJECTS

The subjects were all young married women between the ages of 22 and 34 years living in their own homes, except number II who was a married resident subject at Fels House. Forty-nine balances have been determined by Fels Fund workers using ten subjects. The four subjects whose balances are given in table 1 were selected for comparison with Macy's and Coons' subjects because they had no calcium or phosphorus therapy during pregnancy and their abnormalities seem slight enough to be called intermittent or temporary. Subjects II and IV were primiparae and gave birth to a girl and a boy respectively. Subjects VI, VII and VIII were secundiparae and gave birth to a girl, a boy, and a girl respectively. Infants numbers IV and VI may have been

slightly postmature; the other were called normal term infants. Their weights and physical characteristics will be described later. Subject number IV was found to be somewhat anemic during the fourth month in pregnancy, and at the suggestion of her physician ate more iron-containing foods thereafter to arrest this tendency. Number VI was thought

TABLE 1

Calcium and phosphorus utilization during pregnancy; 24-hour samplings expressed in grams per day

		CALCIUM				PHOSPHORUS			
Subject's number	Weeks pre-delivery	Food	Urinary	Fecal	Balance	Food	Urinary	Fecal	Balance
II	31	1.072	0.256	2.302	—1.486	1.116	0.732	1.523	—1.139
	27	1.058	0.288	2.146	—1.376	1.101	1.435	1.570	—1.904
	11*	1.073	0.372	1.439	—0.738	1.117	0.946	0.866	—0.695
	11*	1.103	0.291	1.277	—0.465	1.149	0.908	0.730	—0.489
	11*	1.104	0.356	1.397	—0.649	1.150	1.052	0.815	—0.717
IV	31	1.400	0.185	1.654	—0.439	1.461	0.848	0.807	—0.194
	27***	1.183	0.241	0.866	+0.076	1.410	0.863	0.615	—0.068
	23	1.266	0.214	1.318	—0.266	1.887	0.915	0.896	+0.076
	19***	1.721	0.260	1.168	+0.293	1.867	0.909	0.659	+0.299
	14	....**	0.212	1.684	....	....	1.230	0.759	....
	7***	1.351	0.213	0.832	+0.306	1.797	0.765	0.500	+0.532
	1***	2.078	0.138	1.594	+0.346	1.489	1.232	0.799	—0.542
VI	23	0.911	0.175	1.511	—0.775	0.885	0.634	0.880	—0.629
	19	1.756	0.183	1.303	+0.270	1.123	0.843	0.566	—0.286
	14***	1.255	0.210	0.898	+0.147	1.036	0.384	0.623	+0.029
	11***	1.591	0.152	1.056	+0.383	1.899	0.910	0.462	+0.527
	7***	1.725	0.252	1.403	+0.071	1.407	0.871	0.776	—0.240
	3	1.578	0.141	1.558	—0.121	1.390	0.686	0.898	—0.194
VII	11***	1.853	0.204	1.229	+0.420	1.397	0.707	0.777	—0.087
	7***	1.856	0.235	0.914	+0.707	1.939	0.858	0.589	+0.492
	1***	1.656	0.484	0.644	+0.528	1.543	0.708	0.375	+0.460
VIII	12***	2.076	0.366	1.400	+0.312	1.590	0.848	0.808	—0.066
	8***	1.009	0.357	1.002	—0.348	1.527	0.656	0.863	+0.008
	4	1.675	0.276	0.834	+0.567	1.414	0.356	0.574	+0.484
	1	1.875	0.214	0.970	+0.691	1.094	0.702	0.701	—0.309

* Three consecutive days.

** Sample lost.

*** Fels balances used in the matched comparisons. The other balances for each subject are included to show the general findings for these individuals.

to be anemic also, and usually suffered from hay fever during August which was her ninth month in this pregnancy. Neither condition was treated. Subject number VII had some albuminuria in the ninth month; number VIII had a persistent mild allergy toward egg and lemons and had suffered mildly from hay fever since childhood. The hay fever appeared a few days before delivery and she received treatment for it as usual.

PROCEDURE

The data were collected during the spring and summer of 1934 on subjects VI, VII and VIII, and on subject IV during the spring and summer of 1933. Periods were begun as a rule on Monday or Tuesday to insure completion during the same week. The women prepared and weighed their food in their own homes under our instruction. They were told to select foods which were representative of their usual meals and to eat, at the same time of day for 3 successive days, the same kind and as nearly the same amount of food as possible. On the third day the subject saved a day's sample of food equivalent either to the amount she ate or to one-half the amount. The feces were marked off with carbon and carmine given by capsule at the beginning of breakfast on the third and fourth days respectively. The women were asked to take a very small amount of water with the capsule. Tap water ingested throughout the day was weighed in grams and a similar quantity was included in the food sampling. A medical examination was made just before the period began and again when it ended, and basal metabolic rate and blood samplings were secured on the morning following the 3-day sampling period. The procedure outlined above was repeated every 28 days when possible, during the whole course of the prenatal period when the women were under observation.

METHODS USED IN ANALYSIS

Food, fecal, urinary, and blood samples collected for mineral analyses were all dried on the steam bath and then ashed

in the muffle furnace at a temperature below 400°C. until a loose, grayish ash was produced. The ash was put into solution with warmed, constant-boiling HCl and redistilled water. The residue was re-ashed and treated again to insure that the mineral was freed from carbon particles. Samplings from this solution made to volume were used for all analyses.

Calcium in food, feces and urine was determined volumetrically in triplicate, observing the precautions cited by Washburn and Shear ('32); phosphorus was determined by the method used by Embden and Fetter ('21).

METHOD OF COMPARISON

As stated in the beginning, the object in making this study was to see how data collected by the 24-hour method of sampling compared with the results presented by Coons ('30, '34) and by Macy ('30) and their co-workers on longer period samplings. Accordingly the following procedure was used. Twelve balances were selected from our data, and twelve each from the first and second studies made by Coons and her co-workers in order to match the twelve given by Macy and her co-workers. Balances determined consecutively on the same subject were used as far as was possible in order to fulfill two criteria for matching; namely, that the four sets of twelve balances should each fall in the same period in pregnancy, and should be representative of approximately the same number of individuals. The tables and the graphs show how close the matching could be made and how the results compare.[2]

[2] The Fels balances used are starred in table 1. Coons' first group ('30) is designated as Coons I and her second group ('34) as Coons II. In her first study (Coons I) all balances for subjects A and D, the third balance for subject B, the first and second balance for C, and the third balance for E were used in the matching. The balances selected from her second study (Coons II) were: the first and second for subject I, all for II, the first three for V, and the first and last two for VI. All of the balances given by Macy and her co-workers ('30) were used here.

From these forty-eight calcium and phosphorus balances taken from the four studies, comparisons were made in the following ways between each group of twelve balances:

a. Means, standard deviations, coefficients of variation and their probable errors for Macy, Coons and Fels balances were computed (table 2).

b. Urinary and fecal partitions for both calcium and phosphorus were calculated by dividing the excretion by the ingestion. Means, standard deviations and coefficients of variability were calculated for the partitions and included in table 2.

c. Pearson's product-moment correlations for food and fecal, and for food and urinary calcium and phosphorus were computed for each of the four groups of matched balances.

d. Diagrams were made showing the distribution of the balances around curves computed by the Method of Least Squares.

e. The findings in (a) and (b) were compared by computing critical ratios (table 3). Our basis for interpreting these ratios is given in the handbook of Chemistry and Physics, eighteenth edition, page 180. When the ratio was less than 2.0 the differences found were called insignificant; between 2.0 and 3.0, somewhat or tending toward significance; when the ratio was 3.0 or over, the difference was called significant.

The means, standard deviations and coefficients of variation in table 2 are given only to clarify the critical ratios. It is obvious that variability should be high for average mineral output in feces and urine because the balances are scattered throughout most of the period of pregnancy. An average for excretion, therefore, computed from consecutive balances which are inevitably modified by rapid growth of the fetus and by the maternal needs is of little use except in matched comparison and where average variation is being considered. The averages for mineral content of the food, however, do show how uniform was the amount of calcium and phosphorus ingested by each of the four groups at various stages in pregnancy.

TABLE 2

Means, standard deviations and coefficients of variation for matched ingestion and excretion values

		MACY	COONS I	COONS II	FELS
Urinary calcium	M[1]	0.447±0.031	0.204±0.017	0.204± 0.021	0.215±0.016
	SD	0.162±0.022	0.087±0.012	0.104± 0.014	0.083±0.011
	C of V	33.9 ±5.19	42.6 ±6.85	60.0 ±10.8	38.6 ±6.05
Fecal calcium	M	1.549±0.092	0.857±0.049	1.034± 0.081	1.076±0.045
	SD	0.474±0.065	0.252±0.035	0.414± 0.057	0.233±0.032
	C of V	30.6 ±4.60	29.4 ±4.38	40.0 ± 6.33	21.7 ±3.13
Food calcium	M	1.922±0.065	1.149±0.058	1.537± 0.092	1.610±0.065
	SD	0.333±0.046	0.299±0.041	0.473± 0.065	0.335±0.046
	C of V	17.3 ±2.45	26.0 ±3.81	30.8 ± 4.63	20.8 ±2.99
Urinary calcium partition	M	0.253±0.017	0.182±0.016	0.128± 0.011	0.170±0.016
	SD	0.085±0.012	0.083±0.011	0.056± 0.008	0.084±0.012
	C of V	33.6 ±5.12	45.6 ±7.47	43.7 ± 7.07	49.4 ±8.30
Fecal calcium partition	M	0.797±0.049	0.736±0.027	0.660± 0.021	0.685±0.028
	SD	0.253±0.035	0.141±0.019	0.109± 0.015	0.144±0.020
	C of V	31.7 ±4.78	19.2 ±2.74	16.5 ± 2.33	21.0 ±3.01
Urinary phosphorus	M	1.241±0.053	0.723±0.024	0.744± 0.033	0.809±0.041
	SD	0.272±0.037	0.124±0.017	0.168± 0.023	0.210±0.029
	C of V	21.9 ±3.16	17.2 ±2.44	21.7 ± 3.13	26.0 ±3.81
Fecal phosphorus	M	0.747±0.037	0.580±0.034	0.59 ± 0.04	0.654±0.029
	SD	0.190±0.026	0.177±0.024	0.19 ± 0.03	0.149±0.021
	C of V	25.4 ±3.72	30.5 ±4.57	32.2 ± 4.87	22.8 ±3.30
Food phosphorus	M	2.248±0.081	1.442±0.063	1.74 ± 0.09	1.575±0.049
	SD	0.415±0.057	0.325±0.045	0.47 ± 0.07	0.252±0.032
	C of V	18.5 ±2.63	22.5 ±3.25	27.0 ± 3.98	16.0 ±2.26
Urinary phosphorus partition	M	0.555±0.014	0.527±0.014	0.452± 0.009	0.516±0.023
	SD	0.070±0.010	0.073±0.010	0.047± 0.006	0.117±0.016
	C of V	12.6 ±1.76	13.9 ±1.95	10.4 ± 1.45	22.7 ±3.28
Fecal phosphorus partition	M	0.575±0.035	0.403±0.017	0.337± 0.015	0.397±0.029
	SD	0.181±0.025	0.087±0.012	0.079± 0.011	0.147±0.020
	C of V	31.5 ±4.75	21.6 ±3.11	23.4 ± 3.39	37.0 ±5.75

[1] In this table M indicates mean, SD indicates standard deviation, and C of V indicates coefficient of variation.

TABLE 3

Critical ratios

	MACY AND COONS I	MACY AND COONS II	COONS I AND COONS II	MACY AND FELS	COONS I AND FELS	COONS II AND FELS
Urinary calcium						
M[1]	6.9 (M)[2]	6.6 (M)	0	5.3 (M)	2.2 (F)	2.0 (F)
SD	3.1 (M)	3.1 (M)	0.9 (II)	2.6 (M)	0.4 (F)	0.5 (C)
C of V	1.0 (C)	2.2 (C)	1.4 (II)	0.3 (F)	0.7 (C)	1.9 (C)
Fecal calcium						
M	6.6 (M)	4.2 (M)	1.9 (II)	4.6 (M)	3.3 (F)	0.5 (F)
SD	3.0 (M)	0.7 (M)	2.4 (II)	3.3 (M)	0.4 (C)	2.4 (C)
C of V	2.0 (C)	1.2 (C)	1.7 (II)	1.6 (M)	1.4 (C)	2.8 (C)
Food calcium						
M	8.9 (M)	3.4 (M)	3.7 (II)	3.4 (M)	5.3 (F)	0.9 (F)
SD	0.6 (M)	1.8 (C)	2.3 (II)	0.0 (F)	0.6 (F)	1.7 (C)
C of V	2.0 (C)	2.6 (C)	0.8 (II)	0.9 (F)	1.2 (C)	1.8 (C)
Urinary calcium partition						
M	3.0 (M)	6.2 (M)	2.8 (I)	3.6 (M)	0.5 (C)	2.2 (F)
SD	0.1 (M)	2.0 (M)	2.0 (I)	0.1 (M)	0.1 (F)	1.9 (F)
C of V	1.3 (C)	1.2 (C)	0.2 (I)	1.6 (F)	0.3 (F)	0.5 (F)
Fecal calcium partition						
M	1.1 (M)	2.6 (M)	2.2 (I)	2.0 (M)	1.3 (C)	0.7 (F)
SD	2.7 (M)	3.8 (M)	1.3 (I)	2.7 (M)	0.1 (F)	1.4 (F)
C of V	2.5 (M)	2.9 (M)	0.8 (I)	1.9 (M)	0.4 (F)	1.2 (F)
Urinary phosphorus						
M	8.9 (M)	7.5 (M)	1.3 (II)	6.5 (M)	1.9 (F)	0.7 (F)
SD	3.6 (M)	2.4 (M)	1.5 (II)	1.3 (M)	2.6 (F)	1.1 (F)
C of V	1.2 (M)	0.0 (M)	1.1 (II)	0.8 (F)	1.9 (F)	0.9 (F)
Fecal phosphorus						
M	3.3 (M)	2.9 (M)	0.2 (II)	1.9 (M)	1.7 (F)	1.4 (F)
SD	0.4 (M)	0	0.4 (II)	1.2 (M)	0.9 (C)	1.2 (C)
C of V	0.9 (C)	1.1 (C)	0.3 (II)	0.5 (M)	1.4 (C)	1.6 (C)
Food phosphorus						
M	7.9 (M)	4.2 (M)	2.7 (II)	7.1 (F)	1.7 (F)	1.6 (C)
SD	1.3 (M)	0.6 (C)	1.8 (II)	2.5 (M)	1.5 (C)	2.8 (C)
C of V	0.9 (C)	1.8 (C)	0.9 (II)	0.7 (M)	1.6 (C)	2.4 (C)
Urinary phosphorus partition						
M	1.4 (M)	6.2 (M)	4.5 (I)	1.4 (M)	0.4 (C)	2.6 (F)
SD	0.2 (C)	2.0 (M)	2.2 (I)	2.5 (F)	2.3 (F)	4.1 (F)
C of V	0.5 (C)	1.0 (M)	1.4 (I)	2.7 (F)	2.3 (F)	3.4 (F)
Fecal phosphorus partition						
M	4.4 (M)	6.3 (M)	2.9 (I)	3.9 (M)	0.2 (C)	1.8 (F)
SD	3.4 (M)	3.7 (M)	0.5 (I)	1.1 (M)	2.6 (F)	3.0 (F)
C of V	1.7 (M)	1.4 (M)	0.4 (II)	0.7 (F)	2.4 (F)	2.0 (F)

[1] M indicates mean, SD indicates standard deviation, and C of V indicates coefficient of variation.

[2] In this table the letter in parenthesis indicates which study had the higher value. For example, 6.9 (M) indicates that Macy's group had the higher value in addition to being of significantly different value than Coons I.

CALCIUM

Ingestion. The figures for average ingestion and excretion are given in table 2, and for the significance of any difference in table 3. Macy's group had the greatest average calcium ingestion, Fels group next, Coons II next and Coons I the least in amount. The critical ratio showed that each of them received a significantly different amount except when Coons II and the Fels group were compared. Although Macy's subjects varied less than the other three in average calcium ingestion, the differences in variability were not significant except when her results were compared with Coons II. The latter group was about 13.5 per cent more variable than the former.

Partition. A number of significant differences appeared when the average calcium partition was calculated. Partition as used here means the figure obtained by dividing the amount of calcium excreted in urine and feces respectively by the amount contained in the food. Means, standard deviations and coefficients of variability were calculated for partitions in each of the four studies. These results were examined with particular interest, because it was felt that there the effects of mineral 'lags' might be revealed if the partitions for the Fels Fund study were decidedly atypical with respect to other values computed for pregnancy.

Possibly because the ingestion values were greater, the average urinary calcium partition for Macy's group was greater than the other three groups. The figure was not significantly more variable than the other three. Coons I, with the next highest value, differs significantly from Macy's group and tends to have a greater partition figure than Coons II. Coons I has a somewhat greater standard deviation, but the variability in Coons' two studies is very similar.

The Fels group ranks between Coons I and Coons II in average urinary calcium partition. In the Macy-Fels pairing, Fels subjects were more variable on the average, and the Coons II-Fels pairing showed that the Fels group had the greater standard deviation. However, the critical ratios for the standard deviations of the latter pairing is 1.9, and for

Coons II-Macy and Coons II-Coons I pairings are each 2.0. Consequently the urinary calcium partition in the Fels group does not seem to be clearly aberrant.

Fecal partition should be influenced by mechanical defects in marking. There is no doubt that this hazard is great in 24-hour sampling. However, the calculations revealed no significant differences in the four studies. Macy's group and Coons II differ most in mean, standard deviation, and coefficient of variation. Coons I and Coons II tend to differ in the mean. Fels' and Macy's groups differ in mean and standard deviation and that difference approaches significance. The Fels group and Coons I and Coons II are quite similar in all respects. On the whole Coons II and Macy's groups differ the most in fecal partition.

PHOSPHORUS

Ingestion. Again the means, standard deviations, coefficients of variation and partitions given in table 2 were studied, and the critical ratios given in table 3 were examined to see whether the groups differed significantly. Macy's group had ingested the most phosphorus on the average. Matched against Coons I and II and the Fels group, this difference in average phosphorus ingestion becomes significant, but the amount ingested is not more variable. Coons II ranks next highest, and those subjects tended to use more variable quantities than the Fels group, which had the third greatest phosphorus ingestion. Coons I had the smallest phosphorus ingestion and tended to differ significantly from Coons II. Coons I and the Fels group are more alike in the quantity of phosphorus ingested than any other paired groups.

Partition. Urinary phosphorus excretion per gram of phosphorus intake shows a higher mean value for Macy's group than for the other three. The difference is not significant except where her findings are paired with Coons II. Macy's average partition resembles Coons I, but there is enough difference in means between Coons I and Coons II

and between Macy's group and Coons II to permit one to conclude that there is as much difference in the average for urinary phosphorus partition in these three studies as there is between any one of them and the Fels 24-hour period samplings judged from the balances selected here. Macy and Coons II tend to differ in standard deviation, but the difference between them in variability is negligible. Fels mean value ranks between the figures for Coons I and II, and has a significantly greater standard deviation and variability than all three of the other groups. The Fels mean tends to differ significantly only when matched against Coons II.

The calculation for fecal phosphorus output per gram of phosphorus intake gives Macy's group the highest value on the average. Her group partition again differs significantly from the other three. The standard deviation is significantly greater than Coons I and Coons II and is slightly greater than the value for Fels group. Coons I differs from Coons II in the mean, but the variability is similar and the range of variability in Coons' subjects is less than Macy and Fels Fund groups.

DISCUSSION[3]

A clear-cut difference between the long and short sampling period for calcium and phosphorus metabolism during pregnancy was not found when the four studies were compared by matching averages, distribution, and range in mineral

[3] Only one subject, subject II (see table 1), has been observed on each day of the 3-day balance period. She had just recovered from a rather severe cold, contracted while taking a long automobile trip during a brief holiday. When she returned, she was asked to eat the same kind of food which she had eaten in December and January and to eat as near the same amount of food for 3 days as she could. It was interesting to note that she was continuously in negative balance. At the beginning of pregnancy she was in poor physical condition as her food intake had been very much restricted. Yet after 4 months of quite intelligent and abundant choice of fresh fruits, vegetables and meat and the use of a quart of milk, cod liver oil and yeast daily, she was still in negative balance. Her gain in weight had been rapid and she had been active and well except for the one cold. The results for the 3 successive days differ, but interestingly enough, either the first day or the third day approaches the average of the 3 days fairly closely in calcium or phosphorus distribution. Calculations for differences on each of the 3 days on other subjects are in progress.

balance figures. For phosphorus the results are less clear-cut than for calcium, but the general evidence seemed to us to point toward differences due to differences in the individuals who were studied rather than to the mechanics of sampling. A further test was applied by examining the correlations made to show the relation between ingestion and excretion for both calcium and phosphorus (see table 4).

TABLE 4

ITEMS CORRELATED	OBSERVER	r	PE_r
Food and fecal calcium	Macy	+0.02	±0.20
Food and fecal calcium	Coons I	+0.84	±0.06
Food and fecal calcium	Coons II	+0.95	±0.02
Food and fecal calcium	Fels	+0.41	±0.16
Food and urinary calcium	Macy	+0.04	±0.19
Food and urinary calcium	Coons I	+0.17	±0.19
Food and urinary calcium	Coons II	+0.01	±0.19
Food and urinary calcium	Fels	—0.03	±0.19
Food and fecal phosphorus	Macy	+0.35	±0.17
Food and fecal phosphorus	Coons I	+0.66	±0.11
Food and fecal phosphorus	Coons II	+0.83	±0.06
Food and fecal phosphorus	Fels	—0.33	±0.17
Food and urinary phosphorus	Macy	+0.55	±0.16
Food and urinary phosphorus	Coons I	+0.86	±0.05
Food and urinary phosphorus	Coons II	+0.92	±0.03
Food and urinary phosphorus	Fels	—0.42	±0.16

Some factor tends to shift the Fels results toward a species of negative relationship—the greater the ingestion, the greater the retention, although the correlation is too low to be used as conclusive evidence. Correlations made on Macy's calcium findings are equivocal, and the correlations for phosphorus are too low to be conclusive. The indication is that the greater the phosphorus ingestion, the greater might be the excretion. On the other hand, Coons' results are quite clearly positive for phosphorus, namely, the greater the ingestion, the greater the excretion, but the results were equivocal for urinary-food calcium correlation. This is interesting and puzzling since Macy's group received the largest amounts of both calcium and phosphorus on the average, and calcium

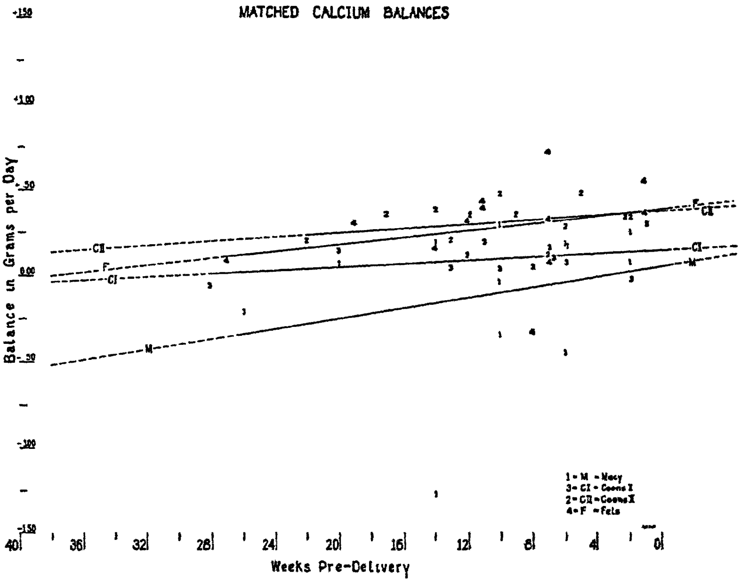

Figure 1

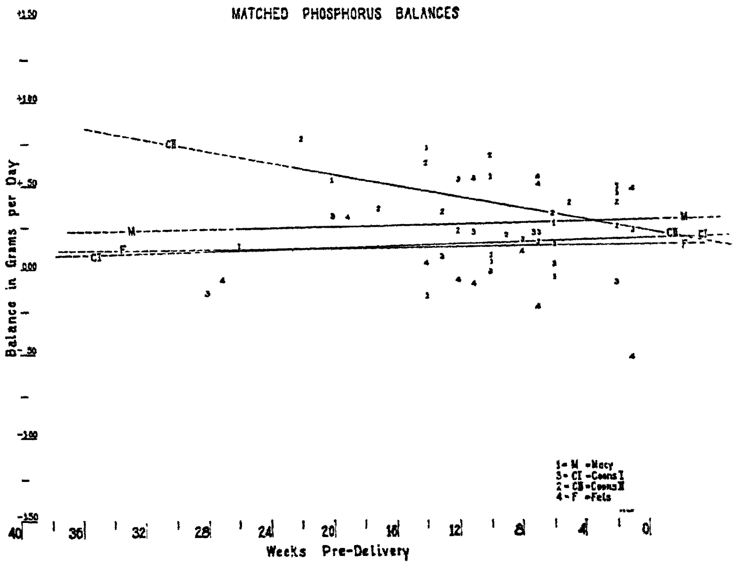

Figure 2

These charts show the distribution and average trends of the four groups of balances discussed in the paper. The curves were computed by the method of least squares.

in significantly greater amounts than the amount received both by Coons I and Coons II.

Finally graphs were constructed to see what the scatter of the forty-eight balances was (see figs. 1 and 2). The results showed that the Fels calcium and phosphorus balances fitted in fairly well with the longer period averages of Macy and Coons. Increased retention which must be a logical state for normal pregnancy is indicated for calcium, and in a lesser but positive degree for phosphorus by these balances. The scatter for phosphorus is less than that for calcium for all four groups of balances charted. Apparently the Fels group stored about 7.0 and 4.5 gm. of calcium and phosphorus respectively during pregnancy. In view of the amount of mineral which is present in the fetus at birth, the demands made upon the maternal stores must have been large in all four groups.

It was interesting to note that the figure for calcium excretion by the kidney route was decidedly more variable than the output by the intestinal route in each of the groups receiving the least amounts of calcium and phosphorus. This is most apparent in the Fels study. We are at a loss for an explanation since the reverse is true for phosphorus partition.

SUMMARY

A comparison was made between twelve calcium and phosphorus balances computed by the 3-day method and those given by Macy and her co-workers ('30), and twelve from each of the studies made by Coons and her co-workers ('30, '34). The balances in the four studies were matched chronologically in order to rule out differences seen at various stages in pregnancy due to the growth of the fetus and changes in maternal tissues.

The results from 24-hour balance sampling preceded by 2 days of preparation when the subject ate the same kind and as near the same quantity of food as on the third (collecting) day did not differ significantly enough from the results given in the other three studies to indicate that a period of this

length fails to show an individual's metabolic tendencies during pregnancy.

The findings point toward greater individual differences than difference due to method.

On the strength of these indications we feel that the 3-day balance period with a 24-hour sampling on the third day merits consideration as a clinical tool in determining human calcium and phosphorus utilization during pregnancy.

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THE POTENTIAL ALKALINITY OF HONEY: ITS ACID-BASE VALUE AS A FOOD

R. E. LOTHROP
Carbohydrate Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture

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No very extensive study of the mineral components of honey has been made, although a number of investigators have carried out limited studies of the composition of the ash of various types of honey. These investigations, however, were mostly of foreign honeys, so that there is actually very little information relative to the composition of American honeys from this standpoint.

The following mineral elements have been found in honey: Phosphorus (Elser, '25, '26), calcium (Elser, '28; Stitz, '27), potassium (Svoboda, '33), magnesium (Jewell, '31), sodium (Nottbohm, '27), chlorine (Nottbohm, '14), sulphur (Nottbohm and Weinhausen, '14), manganese (Gottfried, '11, '12), iron (Schuette and Remy, '32), aluminum (Jewell, '31), copper (Lindow, Elvehjem and Peterson, '29), and silicon (Jewell, '31). The relative proportions in which these elements occur appear to vary widely, although in general phosphorus, calcium and potassium predominate.

Nutrition specialists consider honey almost altogether as an energy food, as it contains usually from 75 to 80 per cent of sugars. Since the average ash content is quite low, and only comparatively small amounts of honey can be eaten at one time the mineral elements have been given very little consideration. However, increasing quantities of honey are now being used for confectionery purposes and in baking, particularly bread, so that much greater quantities may be consumed by an individual in this way.

Although it has been shown that honey yields a decidedly alkaline ash (Nelson and Mottern, '31), the acid-base balance of honey as a food has not been studied. When the alkalinity of the ash is used as a measure of the acid-base balance, correction should be made for the loss of chlorine and sulphur which occurs during ashing. These losses have been pointed out by Nottbohm ('14).

Since the data on the composition of the mineral components of American honeys are too limited to permit calculations of the acid-base balance, the recently published method of Davidson and LeClerc ('35) for determination of the acid-base balance of foods offered a convenient means of obtaining these values for honey. Accordingly, this method was applied to the determination of the acid-base balance of a number of American honeys, including the more representative floral varieties.

Briefly, the method used consists of direct titration of the ash, with corrections for the sulfur and the chlorine lost during combustion. This procedure entails fewer and simpler determinations in comparison with the method based on computation from mineral analyses. Results are approximately the same by either method. Table 1 gives a summary of the results obtained. All the honeys studied gave definite alkaline values. With a few exceptions, values for the dark-colored honeys are somewhat greater than for the lighter-colored varieties, due apparently to the generally higher ash content of the darker types. The alkaline values closely parallel the ash contents. Honey is similar to fruits in that while definitely acid to the taste, due to the presence of free organic acids, as a food it is potentially alkaline. Apparently a considerable part of the metal elements present is combined with organic acid radicals (Nelson and Mottern, '31).

It might be of interest to compare the potential alkalinity of honey with some of the typically alkaline foods as given by Sherman ('32). While the average ash content of honey is comparatively low, the potential alkalinity compares favorably with some of the fruits and vegetables. For example, the average alkaline value for honey is 1.5, while that

for apples is 3.7; for asparagus, 0.8; for lemons, 5.0; for mushrooms, 4.0; for orange juice, 4.5; for potatoes, 7.0; for pumpkin, 1.5; for tomatoes, 5.6; for peas, 3.6; and for turnips, 2.7.

TABLE 1

Potential alkalinity of honeys of a variety of floral types

PREDOMINANT FLORAL SOURCE	COLOR GRADE (PFUND COLOR SCALE)		ASH CONTENT	TITRATION OF ASH (CC. N/10 ACID PER 100 GM. HONEY)	CORRECTION FOR S LOST IN COMBUSTION (CC. N/10 ACID PER 100 GM. HONEY)	CORRECTION FOR Cl LOST IN COMBUSTION (CC. N/10 ACID PER 100 GM. HONEY)	POTENTIAL ALKALINITY (CC. 1 N ALKALI PER 100 GM. HONEY)
	Reading[1]	Classification					
			per cent				
Sweet clover	0.6	Water white	0.04	4.50	None	1.80	0.27
Orange	1.2	Extra white	0.05	6.40	None	1.40	0.50
White clover	3.0	White	0.08	8.60	None	2.00	0.66
Sage	3.1	White	0.07	7.50	None	1.80	0.57
Tupelo	4.0	Extra light amber	0.09	10.25	None	1.90	0.84
Mesquite	4.5	Extra light amber	0.61	52.40	None	20.20	3.22
Catsclaw	8.5	Amber	0.22	21.80	0.43	2.80	1.86
Goldenrod	8.5	Amber	0.16	12.10	0.12	1.50	1.05
Tulip-Poplar	11.5	Dark	0.30	28.70	None	1.90	2.68
Mixed flowers	12.0	Dark	0.51	50.00	3.31	1.00	4.57
Buckwheat	13.5	Dark	0.10	8.20	0.90	3.10	0.42
Averages							
Light honeys[2]	...	....	0.16				1.01
Dark honeys	...	....	0.26				2.12
All honeys	...	....	0.20				1.51

[1] Intensity of color increases with the numerical values (U. S. D. A., '33).
[2] First six samples are classified as light-colored honeys.

SUMMARY

The Davidson-LeClerc method for determining the acid-base value of a food by titrating the ash was applied to eleven samples of honey representative of both the light and dark varieties. The average alkaline value proved to be 1.5. While this is low, it compares favorably with certain fruits and vegetables, values of which are also given for the purpose of comparison.

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AN IMPROVED SYNTHETIC RATION FOR VITAMIN B_4 STUDIES [1]

O. L. KLINE, H. R. BIRD, C. A. ELVEHJEM AND E. B. HART
Department of Agricultural Chemistry, University of Wisconsin, Madison

ONE FIGURE

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The importance of vitamin B_4 as a necessary constituent of the diet is rapidly gaining recognition, and accurate methods for assay of this factor are greatly needed. In a previous publication (Keenan, Kline, Elvehjem and Hart, '33) we described a paralysis which develops in chicks on diets low in vitamin B_4. A ration for assay and further study of the vitamin was proposed which in our preliminary studies was found quite satisfactory. The chick has been used extensively in this work, as well as for studies of other factors in the vitamin B complex, because of its well-known sensitivity to a lack of the water soluble dietary factors. The chick paralysis occurring on the synthetic diet which we have used has been prevented by feeding vitamin B_4 concentrates prepared according to the procedure proposed by Kinnersley et al. ('33) and by Barnes, O'Brien and Reader ('32). This successful use of the Reader concentrate established the identity of the rat deficiency disease (Reader, '29) with that occurring in chicks.

Extensive use of the synthetic diet described in the previous paper revealed seasonal variations in the response of the chicks receiving the basal diet, as well as those fed standard

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supplements containing the vitamin. These seasonal differences were thought to be due to variations in the storage of the factor in the chicks at hatching. However, recent work has shown that the response is also dependent to a large extent upon the vitamin B_4 content of the various constituents of the synthetic diet. Although the constituents were purchased in fairly large quantities, one or more of the products varied quite frequently.

In this paper we wish to report the results obtained when the synthetic diet was modified to reduce vitamin B_4 intake, as well as to increase certain other essential factors, and to describe a procedure for vitamin B_4 studies in chicks which we have found fairly successful.

EXPERIMENTAL

Methods of handling the experimental animals have been described in previous reports (Kline et al., '32; Keenan et al., '33). White Leghorn chicks, day old, were used exclusively in these studies and were obtained from the poultry department or from commercial hatcheries. In the earlier studies groups of ten animals were used, but when testing only slight modifications in the ration, three to four chicks have been used in each group.

Basal ration 441, the ration previously described, was found satisfactory, paralysis occurring at the fourth week in 100 per cent of the animals, until the summer months of 1934. The constitution of ration 441 is given in table 1. The constituents, undoubtedly, varied considerably in the amounts of water-soluble factors carried as contaminants. The crude casein was an acid-precipitated, domestic casein of good grade. Dextrin was made by autoclaving cornstarch for 2 hours, although by this process not all the starch is dextrinized. The salt mixture used was the Steenbock-Nelson ('23) salts 40, modified to contain sufficient amounts of copper, manganese and zinc. Eight per cent Anheuser-Busch dried yeast (strain C) was used in the diet, a level double that required to furnish sufficient vitamins $B(B_1)$ and $G(B_2)$ as shown by previous

TABLE 1
Composition of rations

CONSTITUENTS	R. 441	441A	441H	443	445	445H	446	448	449	450	451	452
Dextrin	53.5	53.5	53.5	57.5	57.5		70.5	71	70	69	64	64
Dextrin—heated 120°C.—24 hours						57.5						
Crude casein	24						12					
Crude casein—autoclaved 5 hours		24		24								
Crude casein—heated 120°C. —24 hours			24									
Crude casein—autoclaved and heated					24	24						
Casein—purified								18	18	18	18	18
Salts 40	2.5	2.5	2.5	2.5	2.5	2.5	2.5					
Salts I								5	5	5	5	5
A. B. yeast	8	8	8	8	8	8	8					
Brewers' yeast									1	2	2	2
Autoclaved liver residue	10	10	10				5	2	2	2	2	2
Autoclaved extracted liver residue				7	7							
Autoclaved extracted heated liver residue						7						
Crude liver extract powder								2	2	2	2	2
B_1 concentrate								5 γ daily				
Lung tissue											5	
H_2O extracted lung tissue												5
Cod liver oil	2	2	2	1	1	1	2	2	2	2	2	2

assay. The vitamin B_4 content of this yeast was very low, since feeding 30 per cent in the synthetic diet did not reduce the occurrence of paralysis in chicks. The 'growth factor' in liver, recently described (Kline et al., '34), was furnished in 10 per cent liver residue that had been autoclaved to destroy any vitamin B_4 remaining after water extraction. Vitamins A and D were supplied in cod liver oil.

During the summer months of 1934, the occurrence of B_4 deficiency in chicks receiving ration 441 was considerably decreased, and the rate of growth approached that of the positive control group which received vitamin B_4. The data in table 2 will show that of thirty animals receiving ration 441, eight failed to exhibit symptoms of the deficiency, while only two died of paralysis before the end of the 6 weeks experimental period. The average weight of these birds, 212 gm., indicated only slight deficiency of the vitamin. This growth is compared in figure 1 with that of a group receiving a vitamin B_4 supplement (18 per cent vacuum desiccated whole liver) in which growth was normal.

With this failure of ration 441 to allow occurrence of paralysis in 100 per cent of the animals, we attempted to modify the ration by heat treatment. In a study of the heat stability of the B complex factors (Keenan et al., '35) it was found that B_4 may be completely destroyed in liver by autoclaving for 5 hours at 120°C., and partially destroyed in a grain diet by heating in a dry oven at 120°C. 24 hours. Casein was considered the most likely carrier of vitamin B_4 as an impurity, since it is a good adsorbing agent for the water-soluble factors of milk. The casein was autoclaved for 5 hours at 120°C., then used in synthetic diet 441A (table 1). This proved an adequate modification for a short time, but over a long period the results were unsatisfactory, and in table 2 the data indicate that 45 per cent of the sixty-six birds which were fed ration 441A developed no deficiency symptoms during the 6 weeks experimental period. The average weight on this diet was 244 gm., a growth greater than that obtained on ration 441.

Treatment of the crude casein by heating dry in an electric oven at 120°C. for 24 hours, and fed in ration 441H (table 1), gave similar results with reference to occurrence of paralysis and rate of growth (table 2).

TABLE 2

Comparison of growth and occurrence of paralysis on synthetic diets

RATION	NUMBER OF CHICKS	AVERAGE WEIGHT 3 WEEKS, GRAMS	AVERAGE WEIGHT 6 WEEKS GRAMS	NUMBER DEAD AT 6 WEEKS	NUMBER SHOWING DEFICIENCY	NUMBER NOT SHOWING DEFICIENCY	SEVERITY OF GIZZARD LESIONS
R. 441 only	30	95	212	2	22	8	—
R. 441 + B_4 supplement	30	134	360	0	0	30	—
R. 441A only	66	102	244	19	36	30	—
R. 441A + B_4 supplement	12	144	378	2	2	10	—
R. 441H only	3	98	200	0	3	0	—
R. 443 only	26	100	217	9	20	6	—
R. 443 + B_4 supplement	17	117	270	4	6	11	—
R. 445 only	5	70	105	0	3	2	—
R. 445 + B_4 supplement	9	122	237	0	1	8	—
R. 445H only	15	66	108	3	8	7	—
R. 445H + B_4 supplement	9	92	250	0	2	7	—
R. 446 only	9	55	74	2	7	2	—
R. 446 + B_4 supplement	12	75	160	1	5	7	—
R. 448 only	13	65	60	11	13	0	Severe
R. 448 + B_4 supplement	12	97	190	1	12 (slight)	0	Variable
R. 449 only	10	74	—	10	10	0	Marked
R. 449 + B_4 supplement	3	117	320	0	1	2	Marked
R. 450 only	12	66	—	12	9	3	Marked
R. 450 + B_4 supplement	6	120	375	0	0	6	Variable
R. 451 only	12	80	190	6	12	6	Slight
R. 451 + B_4 supplement	12	160	390	0	0	12	Normal
R. 452 only	26	78	—	26	26	0	Slight
R. 452 + B_4 supplement	15	147	360	0	0	15	Normal

Further purification of the liver residue was thought advisable. Since the 'growth factor' has been shown to be completely insoluble in water, additional water extraction of the liver residue, with removal of 30 per cent solids, had no

effect on its growth factor potency. This re-extracted residue was then autoclaved 10 hours, and fed in synthetic diet 443 (table 1) in combination with autoclaved crude casein. Again, reference to table 2 will show that this change did not reduce the rate of growth or increase the occurrence of paralysis. The record for the vitamin B_4 supplement used in ration 443 (table 2) indicates that B_4 had not been reduced materially, but that possibly other factors had been injured.

In synthetic ration 445, crude casein, which had been both autoclaved 5 hours and heated 24 hours at 120°C., was fed in combination with re-extracted autoclaved liver residue. Although the rate of growth of birds receiving this diet was markedly reduced, symptoms of B_4 deficiency were not observed in all cases. When vitamin B_4 was added to diet 445 growth was sub-normal. If, in addition to the autoclaved casein, the re-extracted liver residue and dextrin were subjected to heating at 120°C. for 24 hours (ration 445H, table 1), results similar to those with ration 445 were obtained.

With failure of heat treatment as a means of purification of the synthetic diet with respect to vitamin B_4, an attempt was made to modify the ration by reducing the crude casein and autoclaved liver residue constituents to 12 per cent and 5 per cent respectively. It seemed possible that essential nutritional factors, as yet undescribed, were present in the crude casein and liver residue, and were being destroyed by the drastic heat treatment used in diet 445H. We thought it possible to reduce the absolute amount of B_4 in the diet by reducing the casein and liver residue content, and still retain a supply of unknown constituents. Reference to table 2 will indicate that ration 446 (table 1) was unsuccessful with respect to rate of growth and occurrence of paralysis. Little more than maintenance of weight occurred in birds receiving this diet, and the onset of the paralytic symptoms was gradual, giving a chronic type of the deficiency disease. With the addition of 18 per cent whole liver as a B_4 supplement to ration 446, the growth rate was subnormal and paralysis occurred in several of the animals. A comparison is made in the growth charts for these animals presented in figure 1.

A source of uniform chicks. Throughout this work the lack of uniformity among individual chicks was a marked feature, particularly a lack of uniform response in groups of chicks of different hatching, and of different seasons of the year. Storage of nutritional factors in the egg is known to be influenced by the diet of the hen, which varies considerably during the year under ordinary poultry practices. To reduce this variation to a minimum, eggs were selected from a pen of twenty-four laying hens kept at the poultry department under the same conditions throughout the year. These hens were kept inside the brooder house with windows open, and were fed the following ration:

Basal mash

Ground yellow corn	100
Ground whole oats	50
Wheat bran	100
St. wheat middlings	100
Meat scrap	75
Dried skim milk	25
Ground alfalfa hay	50
Salt	5

Ration for hens

	Per cent
Basal mash	39.6
Cod liver oil	0.4
Whole yellow corn	40.0
Whole wheat	20.0

The hens kept under winter conditions throughout the year, and fed a ration relatively low in vitamin B_4, produced chicks with a uniformly low storage of this factor. These chicks were used in all subsequent experiments.

Further purification of the synthetic ration. The ideal synthetic diet is one in which the 'non-purifiable' constituents are reduced to a minimum; however, the cost of vitamin concentrates available is prohibitive for routine assay of a factor such as B_4. Synthetic diet 448 (table 1) was devised with the idea of reducing as much as possible the supplements containing the vitamin B factors, particularly.

In ration 448 the 8 per cent Anheuser-Busch yeast was supplanted by Merck crystalline vitamin $B(B_1)$, 5 micrograms daily, and 2 per cent liver extract powder as a source of vitamin G and flavin (Elvehjem and Koehn, '35). The growth factor supplement, autoclaved liver residue, was reduced to

a level of 2 per cent. Vitamins A and D were furnished in 2 per cent cod liver oil. At this point in our experiments it was found necessary, due to the fine sensitivity of the chick to inorganic salt balance, to replace salts 40 with salts I of the following composition:

Salts I

$NaCl$	335	$Fe(C_6H_5O_7)_2.6H_2O$	55
$K_2HPO_4.3H_2O$	845	KI	1.6
$Ca_2H_2(PO_4)_2.4H_2O$	190	$MnSO_4.4H_2O$	0.7
$MgSO_4.7H_2O$	204	$ZnCl_2$	0.5
$CaCO_3$	600	$CuSO_4.5H_2O$	0.6

Ratio—Ca : P = 1.88 : 1.00

In a synthetic ration containing 5 per cent of salts I and 18 per cent casein the ratio of Ca : P = 1.37 : 1.00.

Crude casein was purified for use in diet 448 by alternate solution in dilute NH_4OH, precipitation with dilute HCl, and washing of the precipitate with water. Five hundred grams of crude casein were dissolved in a 10-gallon volume of distilled water by addition of 500 cc. of 5 per cent NH_4OH. The casein was precipitated by addition of 5 per cent HCl to the isoelectric point of the casein. The supernatant serum was removed by siphoning, and the precipitate was washed in a 10-gallon volume of distilled water. Two additional precipitations were carried out, and the finally washed casein filtered and dried at 50°C. before a fan. The purified casein was fed in diet 448 at the level of 18 per cent, and 71 per cent of dextrin completed the ration.

Of thirteen animals receiving ration 448, all exhibited acute symptoms of B_4 deficiency. Eleven died during the fourth week of the experiment, the remaining two showing a decline in weight during the sixth week. When a vitamin B_4 supplement was added to ration 448, the vitamin $B(B_1)$ which was fed by pipette became a limiting factor, causing decreased food consumption and consequent decreased B_4 intake. All animals receiving ration 448 plus B_4 supplement had slight but definite symptoms of paralysis.

It was necessary to use a less destructible and more constant source of vitamin $B(B_1)$. It was found possible to use a dried brewer's yeast (no. 118) obtained locally, which by assay was known to furnish sufficient vitamin $B(B_1)$ for chicks at a level of 0.5 per cent. This yeast was added to diet 449 at the level of 1 per cent, and to diet 450 (table 1) at the level of 2 per cent, the latter of which proved most satisfactory, as is indicated in table 2. Growth charts of animals receiving diet 450 and diet 450 with added B_4 are presented in figure 1.

The gizzard factor. Routine post-mortem examination of all animals receiving basal diets 448, 449 and 450 consistently revealed the occurrence of lesions in the lining of the gizzard similar to those described by Dam and Schönheyder ('34). According to a system of grading the severity of these lesions, we used the terms normal, slight, marked, severe and very severe. The lesions were found in gizzards of all animals on the above-mentioned diets, and were graded either marked or severe (table 2). From previous experiments we had found that vacuum dried lung tissue has a preventative effect on the gizzard lesions, and levels as low as 5 per cent may be used to ensure a normal lining in the gizzard. Lack of the substance preventing occurrence of lesions in the gizzard has a profound effect upon the growth of these birds. Whether it is a primary or a secondary effect due to lack of absorption of other required factors has not been determined. Further evidence for the essential nature of this substance will be presented in a later paper. It was found necessary, following purification of the casein, to add a source of the factor preventing gizzard lesions.

In synthetic diet 451 this factor was supplied in 5 per cent vacuum desiccated lung tissue. This material replaced an equivalent amount of dextrin. In six of twelve birds receiving this ration the acute type of paralysis was observed during the third week of the experimental period followed by death within 48 hours, while the remaining six exhibited only slight symptoms, and continued their growth throughout the 6-week

period. Gizzard examination revealed only slight erosion areas in the gizzard lining, while with added B_4 (table 2) growth and gizzard lining were normal, and there was complete freedom from paralysis.

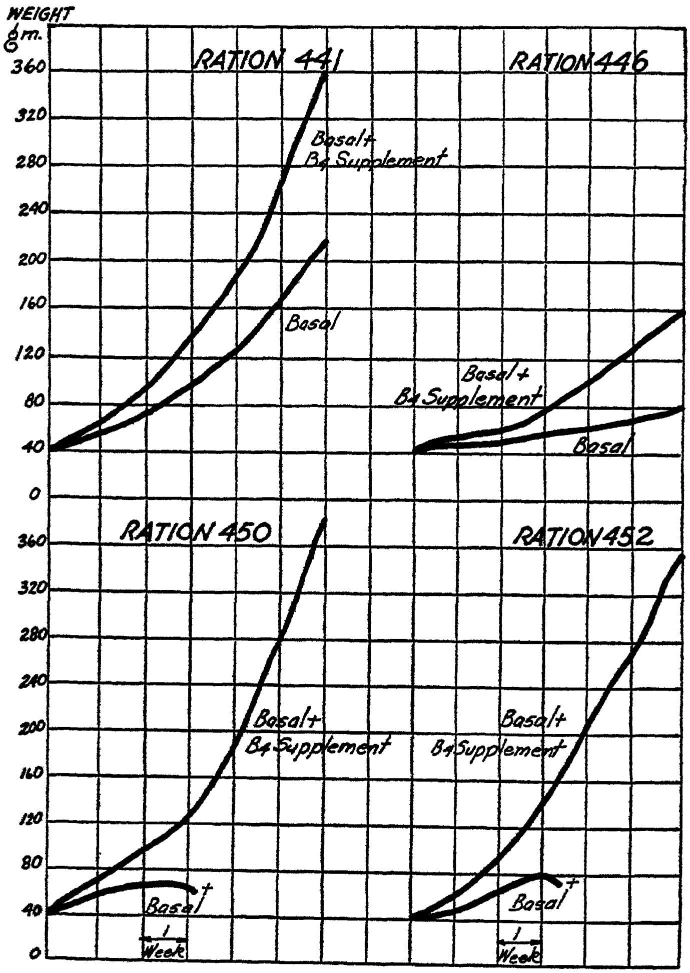

Fig. 1 Representative growth charts for the synthetic diets indicated, demonstrating the development of a successful synthetic diet for vitamin B_4 study in chicks.

A parallel study on the solubility of the factor preventing gizzard erosion showed it to be insoluble in water, giving a method for purifying the lung tissue of its probable vitamin B_4 content. This water extraction of the lung tissue was carried out, with a removal of 25 per cent solids, by extracting the finely ground lung substance three times with hot water. This material, added at the 5 per cent level in ration 452, gave satisfactory results with respect to normal gizzard lining and 100 per cent occurrence of acute vitamin B_4 deficiency. Addition of B_4 to this ration gave normal growth,

TABLE 3

Use of B_4 supplements in ration 452

SUPPLEMENT	NUMBER OF CHICKS	AVERAGE WEIGHT 3 WEEKS, GRAMS	AVERAGE WEIGHT 6 WEEKS, GRAMS	ONSET OF DEFICIENCY, WEEKS	NUMBER WITH DEFICIENCY	DEATHS PRIOR TO 6 WEEKS	SEVERITY OF GIZZARD LESIONS
10 per cent hog kidney	3	107	248	6	2 (slight)	0	Normal
15 per cent hog kidney	3	130	340	—	0	0	Normal
15 per cent hog brain	3	147	365	—	0	0	Slight
5 per cent peanut	6	83	—	3	6	6	Slight
10 per cent peanut	6	108	271	4	6 (slight)	0	Slight
15 per cent peanut	12	143	360	—	0	0	Normal
No supplement	26	78	—	3	26	26	Slight

normal gizzard lining, and complete freedom from paralysis. Constitution of ration 452 is given in table 1. The data for growth and occurrence of paralysis are found in table 2. In figure 1 are presented growth charts for animals receiving ration 452, and 452 with added vitamin B_4 supplement.

An illustration of the use of ration 452 in testing the vitamin B_4 potency of some food materials is given in table 3. Animal tissues were obtained fresh and carefully dried at 50°C. The peanuts used were unroasted, and kept in the shell until needed. They were dried at 50°C. before being ground with the synthetic ration. The rations were compounded

weekly to insure presence of sufficient vitamins A and B_4, factors which are inactivated by oxidation. It is readily seen that 15 per cent of dried brain tissue, dried kidney, or peanuts supplies sufficient vitamin B_4. These supplements at levels of 10 per cent or below were found to be inadequate as a source of vitamin B_4.

DISCUSSION

Our experience with the use of experimental synthetic diets for chicks has brought to our attention a number of interesting facts. Other workers have experienced similar phenomena, and some have been described in the literature, but a re-emphasis is necessary. In the first place, it is impossible to state that one has used a specific ration for the production of a certain nutritional deficiency. All we can say is that a ration has been compounded according to a definite formula. Where the ingredients used in a single laboratory vary from one experiment to another and with each new purchase, it is not surprising that even greater variations occur when the so-called similar rations are compounded in different laboratories. Perhaps one of the greatest offenders in synthetic rations used for both chicks and rats is casein. Casein manufactured at different times of the year certainly must vary when the nutritive value of the milk from which it is made varies. Extraction of casein with various solvents is probably not the most effective means of purifying casein. The particles are still intact and contaminants are not exposed to the solvent. We found that the greatest improvement in our ration depended upon the reprecipitation of the casein. Similar results have been obtained in studies with other vitamins and will be reported later.

Heat has not been found a satisfactory means of purification or separation of factors which do not differ greatly in their heat stability. There occurs an over-lapping destruction which does not give clearly defined results.

The reduction of the yeast in our diet was also a decided advantage. Although yeast is quite low in vitamin B_4, different batches undoubtedly vary in the amount of this factor

which they carry. The introduction of liver extract in place of part of the yeast seems to have a definite beneficial effect, although it is difficult at present to know if the liver extract supplies other essential factors, or if the known factors are more available.

Another fact which is readily evident is that we must give consideration to factors in the B complex other than B_1, B_2, B_4 and flavins. Upon greater refinement of our synthetic diet it has become necessary to add a supplement to prevent the gizzard lesions. Unless this factor is supplied it is impossible to make intelligent studies of vitamin B_4. Paralysis due to poor absorption may occur in chicks receiving ample amounts of B_4 if the chick is suffering from gizzard lesions. This is but another example of the interrelationship of the vitamins.

It is also important to mention that although we speak of ration 452 as one for the production of vitamin B_4 deficiency, it is impossible to conclude that it is complete in all other factors. We know that the addition of 15 per cent peanuts, brain, or kidney as sources of B_4 will produce normal chicks. We have also had fair success with certain B_4 concentrates, but until normal chicks are reared on ration 452 plus pure vitamin B_4 the possibility of still other factors must be considered. We also want to emphasize that ration 452 may not be a perfect ration for B_4 assay. Many improvements may be made in the future, but it is the best available at present.

The variation in the store of factors under question at the time an animal is placed on experiment will probably receive more and more attention. Studies on vitamins B(B_1) (unpublished data) and D (Kline, Elvehjem and Halpin, '35) have demonstrated that feeding varying amounts of these factors to hens has no appreciable effect upon the rate at which chicks produced from these hens develop the respective deficiency diseases when placed on diets very deficient in these factors. It is evident from our work, however, that the vitamin B_4 storage of chicks from hens fed supplements high in this vitamin may be considerably increased. Somewhat similar results have been obtained in the case of the rat.

SUMMARY

1. A revised method for the study of vitamin B_4 in chicks has been described.

2. Changes in a synthetic ration for these studies, previously described, include purification of the casein and use of highly potent sources of the factors in the B complex other than B_4.

3. It was found necessary to add to the ration a substance which prevents the occurrence of lesions in the lining of the gizzard of the experimental chicks. Dried lung tissue is a potent source of this 'gizzard factor.'

4. On the improved basal synthetic ration a nutritional paralysis occurs in 100 per cent of the animals, which may be prevented by addition of supplements such as peanuts, brain, kidney or liver tissue.

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PELLAGRA-LIKE SYNDROME IN CHICKS

S. ANSBACHER, G. C. SUPPLEE AND R. C. BENDER
The Dry Milk Company, Inc., Research Laboratories, Bainbridge, New York

ONE FIGURE

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As early as 1931, Ringrose and co-workers ('31) described the occurrence of a pellagra-like syndrome in chicks and later Kline et al. ('32–'33) reported "a method for the production of definite symptoms of pellagra in the chick" (Elvehjem and Koehn, '35). This method was successfully used in vitamin G (B_2) studies by Elvehjem and Koehn ('35) as well as by Stare ('35). Lepkovsky and Jukes ('35), however, conclude that the syndrome produced by the method of Kline is not due to a deficiency in "vitamin G, the pellagra-preventive (P-P) factor of Goldberger and co-workers ('28)." In view of these publications it seems desirable to report the following data obtained at these laboratories with chicks, wherein the observations are considered in parallel with studies of the dermatitis in rats (Bender et al., '36 and Ansbacher et al., '36).

EXPERIMENTAL

The chicks used were day-old single comb white Leghorns weighing from 35 to 45 gm. The experimental groups contained from fifteen to twenty individuals housed in wire screen compartments 24 × 26 × 18 inches, electrically heated and equipped with raised wire screen (3 mesh to the inch) bottoms. The basal rations were fed ad libitum and the various supplements were administered daily with a pipette.

Weights and observations of pathological symptoms were recorded twice a week. The data reported hereinafter were taken from comparable studies carried out over a period of about 7 months and involving approximately 800 chicks.

The basal ration no. 240-H proposed by Kline et al. ('32–'33) was employed. This ration consists of a mixture containing commercial casein, 12 parts; wheat middlings, 25 parts; yellow corn, 58 parts; and salt (common), 1 part; this mixture is heated to 95–100°C. for 144 hours and then cod liver oil, 2 parts, and calcium carbonate, 2 parts are added. Inasmuch as numerous data have accrued at these laboratories showing the variable character of the results obtained from vitamin studies wherein caseins of unknown history and different origin have been used, Kline's ration was modified for many of the comparative studies reported in the following. This modification consisted of heating the grain mixture in the absence of the commercial casein and substituting vitamin-free casein (Labco)[1] for the commercial casein. The unheated vitamin-free casein was mixed with the other components of the ration after such components as the grains and salt had been heated.

The results obtained from the unsupplemented basal ration containing the heated commercial casein and those obtained from the unsupplemented ration containing the unheated vitamin-free casein are shown in figure 1, graph 1. At the end of 5 weeks, 88 per cent of the chicks receiving the former ration showed the typical pellagra-like syndrome observed by various authors previously cited; 93 per cent of those receiving the unheated vitamin-free casein also showed the typical syndrome in severe form. These data would seem to indicate quite conclusively that the unheated vitamin-free casein (Labco) is fully as effective in permitting the development of the syndrome as the commercial casein heated to about 100°C. for 6 days. It is to be noted, however, that the rate of growth of the chicks receiving the heated commercial

[1] The Labco vitamin-free casein is distributed by The Casein Company of America, Inc., New York.

casein was somewhat greater than that of those receiving the unheated vitamin-free casein, notwithstanding the fact that the incidence and severity of the pellagra-like syndrome was substantially the same for both groups.

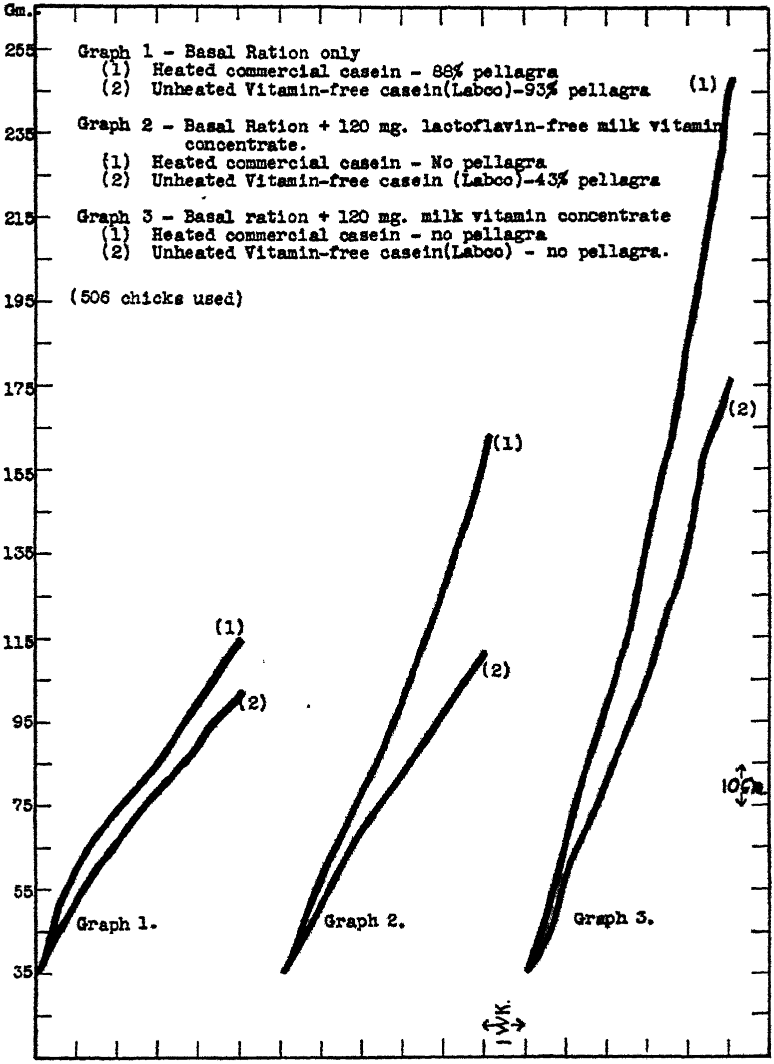

Fig. 1 The influence of commercial and vitamin-free casein on the rate of growth and incidence of the pellagra-like syndrome in chicks.

The significance of the difference in vitamin character of the two caseins is further illustrated by the results obtained from the comparative studies summarized in graphs 2 and 3 of figure 1. The milk vitamin concentrate, reported by Ringrose et al. ('31) to have prevented the onset of the pellagra-like syndrome in chicks, was fed at the 120-mg. level as a daily supplement to chicks receiving the basal rations containing the two types of casein (fig. 1, graph 3). The milk vitamin concentrate rendered lactoflavin-free by treatment with fullers' earth was similarly fed to numerous experimental groups of chicks receiving the two types of casein (fig. 1, graph 2). It will be noted that the rate of growth was considerably less with the unheated vitamin-free casein rations than with the heated commercial casein rations. Furthermore the milk vitamin concentrate at the 120-mg. level prevented the dermatitis irrespective of the caseins used in the basal rations. However, the lactoflavin-free material fed at the same level afforded protection only when the basal ration contained the heated commercial casein. When the basal ration contained the unheated vitamin-free casein, 43 per cent of the chicks manifested the pellagra-like syndrome of the same character as that observed from the feeding of the unsupplemented basal rations. It may be stated parenthetically that the lactoflavin-free material at the same level has been found not to prevent the occurrence of dermatitis in rats receiving a basal ration containing sucrose as the carbohydrate (Bender et al., '36).

These observations seem to show that the heated commercial casein although capable of giving consistent results in the study of the incidence of the pellagra-like syndrome in chicks, nevertheless contains a growth-promoting factor not contained in the vitamin-free casein (Labco). The unknown conditions concerned in the precipitation and production of commercial caseins are such that there is bound to be a substantial and variable retention of the water-soluble factors contained in the original milk. Inasmuch as the milk vitamin concentrate used in these studies gives results compatible in

respect to growth promotion with those obtained from commercial caseins, it is proper to assume that commercial caseins carry a substance or substances of the same character as those contained in the milk vitamin concentrate. The data indicate that such a factor or factors are heat-stable and growth-promoting. Furthermore it appears that fullers' earth removes at least a part of the antipellagra factor carried by the milk vitamin concentrate, since the fullers' earth treated

TABLE 1

Influence of vitamin B (B_1), lactoflavin and rice polish concentrate on growth and incidence of pellagra-like syndrome in chicks fed a basal ration containing unheated vitamin-free casein

SUPPLEMENTS	NUMBER OF CHICKS	PELLAGRA-LIKE SYNDROME INCIDENCE	AVERAGE WEIGHT AFTER	
			3½ weeks	5 weeks
		per cent		
None	83	93	87	102
12.5 γ vitamin B	16	88	97	...
10.0 γ lactoflavin	31	87	94	...
12.5 γ vitamin B and 10.0 γ lactoflavin	16	94	93	...
5 mg. rice polish concentrate	16	75	81	102
25 mg. rice polish concentrate	17	70	88	100
25 mg. rice polish concentrate + 7.0 γ lactoflavin	16	69	92	109
50 mg. rice polish concentrate	17	53	103	123
120 mg. rice polish concentrate	17	0	107	137
120 mg. rice polish concentrate + 7.0 γ lactoflavin	18	0[1]	115	150
240 mg. rice polish concentrate	17	0	97	135

[1] A small percentage of these chicks showed a very mild form of the pellagra-like syndrome.

concentrate, when supplementing the unheated vitamin-free casein ration, protected chicks only if fed at more than twice the level at which the concentrate not so treated afforded full protection.

In view of the above results the basal ration containing the unheated vitamin-free casein was used for the studies summarized in table 1. Crystalline vitamin B (B_1), fed as a daily supplement at the 12.5 γ level, had no significant influence on the incidence of the pellagra-like syndrome,

neither had 10.0 γ of crystalline lactoflavin nor the combination of these two crystalline products. However, the rice polish concentrate, previously shown to protect and cure rats from dermatitis (Bender et al., '36 and Ansbacher et al., '36), was found to contain the factor or group of factors which prevents the onset of the syndrome in chicks. At the 50-mg. level this concentrate protected about one-half of the experimental chicks; when fed in amounts of at least 120 mg., it afforded full protection. The influence of lactoflavin on the rate of growth is indicated in the experiments in which as little as 7.0 γ of crystalline lactoflavin was fed in conjunction with either 25 mg. or 120 mg. of the rice polish concentrate.

The results obtained from the use of the unheated vitamin-free casein (Labco) in place of the heated commercial casein in Kline's ration, seem to be of particular significance in directing attention to the basic importance of the character of the caseins used in studies concerning the various entities of the vitamin B-complex. If it were possible at the present time to determine with surety the exact biological value of the various caseins which have been employed for the various investigations reported heretofore, it is quite probable that many of the apparent discrepancies could be readily reconciled. The results reported herein seem to confirm the early observations of Goldberger and associates ('28) that the P-P factor can be adsorbed on fullers' earth. Lepkovsky and Jukes ('35) consider the possibility of a multiplicity of factors having antipellagric properties, because they and other investigators (Elvehjem and Koehn, '35; Stare, '35) were unable to confirm Goldberger's work. In reality these apparent discrepancies might conceivably be due in part at least to the character of the basal rations used particularly in respect to their casein constituent.

SUMMARY

The use of an unheated vitamin-free casein yielded consistent evidence in showing that the heated commercial casein as a constituent of the 240-H ration of Kline et al. ('32–'33) contains a factor or factors which accelerate the rate of growth and diminish the incidence of the pellagra-like syndrome in chicks.

Concentrates prepared from milk and from rice polish contain a factor or group of factors which prevents the onset of the pellagra-like syndrome in chicks.

Crystalline preparations of vitamin B and of lactoflavin do not prevent the development of the syndrome in chicks.

The factor or group of factors preventing the pellagra-like syndrome in chicks may be adsorbed on fullers' earth.

The data as a whole indicate that the factor or group of factors which prevents and cures the dermatitis in rats (Bender et al., '36 and Ansbacher et al., '36) is identical with that which prevents the pellagra-like syndrome in chicks.

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A STUDY OF THE SEASONAL VARIATION OF VITAMIN D IN NORMAL COW'S MILK [1]

H. ERNEST BECHTEL AND C. A. HOPPERT
Department of Chemistry, Michigan State College, East Lansing, Michigan

THREE FIGURES

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In recent years a great deal of attention has been given to the importance of vitamin D in the human dietary and particularly to the desirability and means of enhancing the milk supply in this factor. The methods by which the latter has been achieved are well known and relatively little thought is now given to the antirachitic potency of milk produced under ordinary farming conditions. Although many studies have been made of the vitamin D content of cow's milk, none of them has been of a comprehensive nature. Table 1 summarizes the results of some of these investigations.

Similar trends in variations of the vitamin D potency of milk are indicated by the work of other investigators. Luce ('24) found milk from pastured cows definitely richer in vitamin D than that from stall-fed cows kept in the dark. Luce concluded that the concentration of the antirachitic factor in milk depended on the ration and possibly also on the degree of illumination received by the cow. Chick and Roscoe ('26) found 0.25 gm. of milk fat from pastured cows equal to 0.60 gm. milk fat from cows fed green feed in a dark stall. Exposure of the cow to outdoor light without a change in ration resulted in a twofold increase in the vitamin D content of

[1] The data in this paper were taken from the thesis presented by H. Ernest Bechtel to the faculty of the Graduate School of Michigan State College in 1935 in partial fulfillment of the requirements for the degree of doctor of philosophy.

the milk fat. Dutcher and Honeywell ('27) examined some Kansas butter samples and found that milk fat from cows exposed to sunshine was superior in vitamin D potency to fat produced by cows fed in the dark.

These and other studies which might be mentioned indicate in a general way the variations that occur in the vitamin D potency of milk and the factors which contribute to this variability. However, most of the work done has been of a rather fragmentary nature so that it seemed desirable to make a more extended study of the subject.

TABLE 1

SOURCE OF DATA	VITAMIN D CONTENT OF NORMAL MILK UNITS PER QUART		
		U.S.P.[1]	Steenbock
Steenbock and associates (Wisconsin) ('30)	June 1925	21.3	7.9
	September 1925	36.5	13.5
Mitchell and associates (Pennsylvania) ('32)	Summer milk	16.2	6.0
Krauss (Ohio) ('33)	Winter milk	7.6	2.8
	Summer milk	16.2	6.0
Russell (New Jersey) ('33)	Summer milk		
	Always less than	43.2	16.0

[1] The values in this column were obtained by multiplying the number of Steenbock units by 2.7.

MATERIALS AND METHODS

This study was begun in 1932, the milk being derived from various sources. Milk from the Holstein and Guernsey herds at Michigan State College was assayed monthly over a period of 2 years. Only the higher producers, namely, cows that were being milked three times daily were used, the average number of Holstein and Guernsey cows being fourteen and eight, respectively, for the 2-year period. From these cows 100-pound portions of composite 24-hour samples were saved monthly.

Monthly samples of a similar nature were derived from a few of the highest producing cows in the Holstein herd of the Michigan Experiment Station. These cows were kept on a ration of alfalfa hay, silage and corn.

From July 1933 to July 1934 monthly samples of Michigan State College Creamery butter, made from milk produced by local Michigan dairymen, were also put aside for vitamin D assays. Approximately fifteen herds, consisting largely of grade Holstein cows were represented in this group of samples.

In the case of the milk a small portion was used for a fat determination, the remainder being run through a cream separator. The butter obtained by churning the cream was packed in paper cartons and stored at 0°F. until the samples were to be assayed. At this time the butter was heated on a steam bath for about an hour and the relatively pure milk fat upon which the assays were conducted was suctioned off. Except when needed for assay, the fats were kept at 0°F.

The vitamin D determinations were carried out by the curative feeding technic, several changes being made in the official procedure. The Steenbock basal ration was slightly modified to obtain more consistent and somewhat better growth of the rats during the preliminary period. The rachitogenic diet used throughout this study was composed of the following:

	Per cent
Yellow cornmeal	38.0
Oatmeal	37.5
Wheat gluten	20.0
Calcium carbonate	3.0
Sodium chloride	1.0
Yeast powder	0.5

Instead of feeding the fat as a daily supplement during the first 8 days of the test period, the entire amount was mixed with 40 gm. of the basal diet. This mixture was found to be consumed in 6 to 8 days after which the basal diet was fed to finish the 10-day period. Control rats receiving 29 mg. of Official Reference Oil equivalent to 2.7 U.S.P. units were used for comparison. The usual staining technic was applied to the radii and ulnae.

In carrying out the assays a preliminary test was made to determine the approximate vitamin D content of the vari-

ous samples. The confirmatory tests were then conducted at three levels using three to five rats at each level.

It became apparent early in this investigation that fats of low potency could not be assayed because of the limited capacity of the rats to consume fat. Amounts up to 6 and 8 gm. were consumed fairly consistently. However, when the dosage was increased to 10 gm. approximately only half of the test animals consumed the fat-basal diet mixture in 8 days. Attempts were therefore made to effect a concentration of the vitamin D so that fats of lower potency might be assayed. Although the work of Kon and Booth ('34) indicated that at least a part of the vitamin D of butter fat was unstable and could not be recovered quantitatively in the non-saponifiable matter, their method as well as several modifications were given a trial. Sometimes the recovery of vitamin D was quantitative, but more often it was not, so that this method of concentration was abandoned.

Inasmuch as the concentration of vitamin D in cod liver oil may be accomplished by extraction of the oil with alcohol, this method was next tried and proved to be satisfactory. The fats of low potency were therefore treated in the following manner: 100 gm. of melted milk fat was placed in a separatory funnel previously warmed in a 37°C. oven and 100 cc. of hot ethyl alcohol (95 per cent) was added. The mixture was then shaken fairly vigorously and the funnel placed into the 37°C. oven until the layers had separated. The fat was then drawn off into a warm beaker and the alcohol layer into a 500 cc. volumetric flask. The fat was returned to the separatory funnel and the beaker rinsed with 50 cc. of hot alcohol which was then added to the fat. The mixture was again shaken and the layers allowed to separate in the oven. The separations were made as before and three additional extractions carried out with 50 cc. portions of hot alcohol. By this process approximately 20 per cent of the fat was removed and this fat contained all of the vitamin D. The combined extracts were brought to a volume of 500 cc. with ether in order that the fat would be kept in solution. It was observed

that the antirachitic value was retained for at least 2 months when the extract was stored at 0°C. Aliquots of this solution were poured on 40 gm. portions of the rachitogenic diet in evaporating dishes and the ether and alcohol allowed to evaporate spontaneously. These mixtures were then fed in the usual manner.

TABLE 2

Antirachitic potency of milk fat obtained from the Holstein herd[1]

DATE	FAT-CONTAINING 1 U.S.P. VITAMIN D UNIT	AVERAGE DAILY PRODUCTION PER COW		FAT IN MILK	VITAMIN D PER QUART OF MILK		AVERAGE DAILY AMOUNT OF SUNSHINE
		Fat	Vitamin D		U.S.P.	St.[2]	
	gm.	*gm.*	*U.S.P.*	*per cent*			*hours*[3]
1932							
July	1.5	817	545	3.26	17.8	6.6	11.5
August	1.1	717	701	2.88	20.2	7.5	10.0
September	1.1	862	784	3.53	27.7	10.3	7.8
October	3.3	748	227	2.78	6.3	2.3	3.3
November	—	—	—	—	—	—	4.0
December	—	—	—	—	—	—	3.0
1933							
January	5.6	826	148	3.15	4.6	1.7	4.1
February	9.3	875	94	3.26	3.1	1.1	7.1
March	3.7	835	226	2.97	6.7	2.5	4.4
April	3.7	748	202	2.93	5.9	2.2	6.8
May	3.0	730	243	2.89	7.0	2.6	8.5
June	—	—	—	—	—	—	12.7
July	1.5	776	517	3.20	16.6	6.1	12.8
August	1.9	862	454	3.28	14.9	5.5	10.8
September	1.5	780	520	2.98	15.5	5.7	7.1
October	3.7	690	186	2.96	5.5	2.0	6.0
November	7.4	753	102	3.19	3.2	1.2	2.0
December	—	—	—	—	—	—	1.4
1934							
January	—	—	—	—	—	—	2.3
February	7.4	844	114	3.17	3.6	1.3	5.9
March	7.4	939	127	3.29	4.2	1.6	5.0
April	4.4	871	198	3.06	6.1	2.3	6.0
May	3.0	898	299	3.31	9.9	3.7	11.3

[1] Average of fourteen cows per month.

[2] Steenbock.

[3] Average amount of available sunshine according to the East Lansing Weather Bureau.

The above method permitted practically complete recovery of the vitamin D from samples of milk fat of which 2 to 10 gm. had to be fed of the original fat to get the typical narrow continuous line of calcification. Inasmuch as there was no way of checking the fats of lower potency directly, it had to be assumed that the method was also satisfactory for such

TABLE 3

Antirachitic potency of milk fat obtained from the Guernsey herd[1]

DATE	FAT-CONTAINING 1 U.S.P. VITAMIN D UNIT	AVERAGE DAILY PRODUCTION PER COW		FAT IN MILK	VITAMIN D PER QUART OF MILK		AVERAGE DAILY AMOUNT OF SUNSHINE
		Fat	Vitamin D		U.S.P.	St.[2]	
	gm.	*gm.*	*U.S.P.*	*per cent*			*hours*[3]
1932							
July	1.1	649	590	4.93	43.8	16.2	11.5
August	1.1	685	623	4.86	43.1	16.0	10.0
September	1.3	708	545	5.15	38.7	14.3	7.8
October	3.0	699	233	4.52	14.7	5.4	3.3
November	3.7	708	191	4.02	10.6	3.9	4.0
December	3.7	930	251	4.76	12.6	4.7	3.0
1933							
January	4.4	907	206	4.42	9.8	3.6	4.1
February	9.3	835	90	4.57	4.8	1.8	7.1
March	3.7	762	206	4.33	11.4	4.2	4.4
April	3.0	658	219	4.34	14.1	5.2	6.8
May	3.0	694	231	4.42	14.4	5.3	8.5
June	—	—	—	—	—	—	12.7
July	1.5	744	496	4.43	28.8	10.7	12.8
August	1.9	853	449	5.09	26.2	9.7	10.8
September	1.9	694	365	4.61	23.7	8.8	7.1
October	2.6	721	277	4.76	17.9	6.6	6.0
November	3.7	912	246	4.86	12.8	4.7	2.0
December	—	—	—	—	—	—	1.4
1934							
January	—	—	—	—	—	—	2.3
February	7.4	866	117	4.44	5.9	2.2	5.9
March	5.6	835	149	4.50	7.8	2.9	5.0
April	3.7	826	223	4.52	11.9	4.4	6.0
May	3.0	871	290	4.90	15.9	5.9	11.3

[1] Average of eight cows per month.

[2] Steenbock.

[3] Average amount of available sunshine according to the East Lansing Weather Bureau.

TABLE 4

Antirachitic potency of milk fat obtained from the experiment station herd[1]

DATE	AVERAGE DAILY FEED INTAKE			FAT CONTAINING 1 U.S.P. VITAMIN D UNIT	AVERAGE DAILY PRODUCTION PER COW		FAT IN MILK	VITAMIN D PER QUART MILK		DAILY AMOUNT SUNSHINE
	Alfalfa	Silicon	Corn		Fat	Vitamin D		U.S.P.	St.[2]	
	lb.	*lb.*	*lb.*	*gm.*	*gm.*	*U.S.P.*	*per cent*			*hours*[3]
1933										
July	24.2	—	15.1	1.3	544	418	2.30	17.3	6.4	12.8
August	26.3	—	13.8	1.5	617	411	2.64	17.2	6.4	10.8
September	26.4	5.5	8.3	1.5	581	387	3.18	20.7	7.7	7.1
October	23.1	25.9	4.4	2.6	508	195	3.34	12.5	4.6	6.0
November	26.0	20.9	3.5	3.7	662	179	4.02	10.6	3.9	2.0
December	24.9	24.0	4.1	3.7	435	118	3.29	8.7	3.2	1.4
1934										
January	—	—	—	—	—	—	—	—	—	2.3
February	26.1	26.0	0.3	4.4	327	74	3.40	7.5	2.8	5.9
March	21.2	19.7	3.2	4.4	531	121	3.73	8.3	3.1	5.0
April	23.8	13.0	9.3	3.7	535	145	2.84	7.5	2.8	6.0
May	26.0	3.6	12.6	2.2	794	361	3.13	13.9	5.1	11.3

[1] Average of five cows per month.

[2] Steenbock.

[3] Average amount of available sunshine according to the East Lansing Weather Bureau.

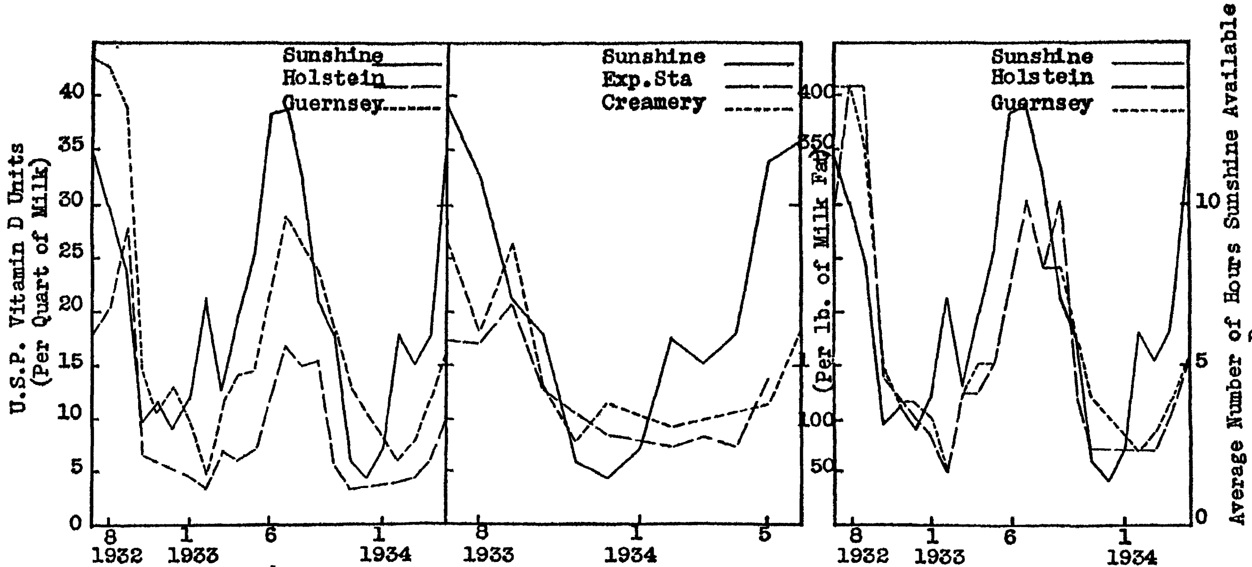
TABLE 5

Antirachitic potency of creamery milk fat[1]

DATE	FAT CONTAINING 1 U.S.P. VITAMIN D UNIT	FAT IN MILK	VITAMIN D PER QUART OF MILK		AVERAGE DAILY AMOUNT OF SUNSHINE
			U.S.P.	Steenbock	
	gm.	*per cent*			*hours*[2]
1933					
July	1.3	3.5	26.3	9.7	12.8
August	1.9	3.5	18.0	6.7	10.8
September	1.3	3.5	26.3	9.7	7.1
October	2.6	3.5	13.1	4.9	6.0
November	4.4	3.5	7.8	2.9	2.0
December	3.0	3.5	11.4	4.2	1.4
1934					
January	—	—	—	—	2.3
February	3.7	3.5	9.2	3.4	5.9
March	—	—	—	—	5.0
April	—	—	—	—	6.0
May	3.0	3.5	11.4	4.2	11.3
June	1.9	3.5	18.0	6.7	11.8

[1] Average of twelve herds of cows per month.

[2] Average amount of available sunshine according to the East Lansing Weather Bureau.



1 2 3

Fig. 1 Antirachitic potency of milk from the Holstein and Guernsey herds, and available sunshine.
Fig. 2 Antirachitic potency of milk from the Experiment Station herd and M. S. C. Creamery, and available sunshine.
Fig. 3 Antirachitic potency of milk fat from Holstein and Guernsey herds, and available sunshine.

fats. Quantitative recovery was also obtained when a definite amount of vitamin D from the official reference oil was added to milk fat and subjected to hot alcohol extraction.

The results of this study of the seasonal variation in the vitamin D content of cows' milk are presented in tables 2, 3, 4 and 5 and portions of the data are shown graphically in figures 1, 2 and 3. The data include not only the results of the bioassays but also the average daily production of milk fat and the number of vitamin D units in the milk fat. The results were also calculated in terms of U.S.P. units per quart and these values are presented in figures 1 and 2 with the average daily hours of sunshine available each month. To simplify the comparison of these results with those given in the older literature the antirachitic potency is also expressed in terms of Steenbock units.

Inasmuch as exposure of the cows to sunlight and the ingestion of sun cured roughages are two important factors which influence the vitamin D potency of the milk, a brief reference to the general management of the several dairy herds is appropriate. The main Holstein and Guernsey herds of the college were kept under parallel conditions at all times. From May to September, inclusive, these animals were pastured an average of 8 hours daily and received no hay or corn silage. During October they were pastured an average of 5 hours daily and received about 1 pound of hay per 100 pounds of body weight. From November to April, inclusive, the animals were put out doors in dry lot for an average of 2 hours daily. During this period in 1932–1933 they received besides their allowance of grain an average of approximately 2 pounds of hay per 100 pounds of body weight, no corn silage being included. During the corresponding period in 1933–1934 the animals received $\frac{3}{4}$ pound of hay and 3 pounds of corn silage per 100 pounds of body weight in addition to grain. The average weight of the Guernsey cows was 1150 and that of the Holsteins 1400 pounds.

The Holstein cows in the experiment station herd were out of doors in dry lot an average of 7 hours daily from May

to September, inclusive, and about 2 hours daily during the other months. These cows were kept on a ration of alfalfa hay, corn silage and corn as shown in table 4.

The general management of the local Michigan dairy herds which served as the source of the college creamery butter samples was typical of that practiced in this state. The cows were fed chiefly home grown feeds consisting largely of alfalfa and cereal grains and were usually pastured as early and as late as conditions permitted. They were probably exposed to sunshine for a longer time than the cows in the college herds.

Regarding the assaying of the various samples of butter, there was a considerable interval between the time the samples were obtained and the time the bioassays were made. This delay was due chiefly to the fact that a satisfactory method had to be developed before the samples of low vitamin D potency could be assayed. However, there appeared to be no danger of a loss of antirachitic potency because some of the older samples were assayed 30 months after the first test was completed, the results indicating that vitamin D in milk fat is stable for at least 30 months when the samples are stored at 0°F. in the dark.

Practically all of the results given in tables 2, 3, 4 and 5 were obtained by using the alcohol extraction method, although most of the summer samples and a few of the more potent winter samples were also assayed by feeding the original fat.

DISCUSSION

In this investigation two assumptions were made which appear to be justifiable. It was assumed that all of the antirachitic potency of cows' milk is present in the milk fat and that there was no significant loss in potency incidental to the separating and churning of the cream.

The standard curative feeding technic was selected for the bioassays because this method has a number of definite advantages over the prophylactic procedure. Besides the fact that the former is much more widely used, it permits the

feeding of relatively large amounts of fat without interfering with the test itself. In the prophylactic method the addition of vitamin D free fat to the basal rachitogenic diet will of itself cause a definite increase in the ash content of the bones, the increase depending on the amount of fat added. In this connection the slight modification of the rachitogenic diet seems justified because the rats attained a slightly larger size at the end of the preliminary period and had somewhat better appetites. This permitted the feeding of larger amounts of fat which was necessary in the case of the samples of lower potency. Nevertheless there were limitations in the capacity of the rachitic rats to consume fat and this necessitated concentration of the vitamin D. The alcohol extraction method described above seemed to solve this difficulty.

Inasmuch as Kon and Booth ('34) had felt that the vitamin D in winter milk might be different from that in summer milk because of the difference in stability to saponification, some of the more potent winter samples were assayed both by feeding the original fat and an equivalent amount of the extract. Although many of the rats failed to consume the larger doses of fat during the first 8 days of the experimental period, a sufficiently large number was used so that an assay at the 10-gm. level was made possible. The results indicated that there was no apparent loss in vitamin D in making the alcohol extractions of the winter samples tested, and served as the basis for the assumption that the assays of fats of still lower vitamin D potency by this method of concentration might be reliable.

The results obtained demonstrate that milk produced by cows managed under practical farming conditions varied as much as 900 per cent in antirachitic potency, reaching a maximum from June to September and beginning with October, declining rapidly to a minimum which usually occurred in February. From the assays made on the milk fats it was calculated that the maximal potency of the milks examined in this study was 43.8 U.S.P. units per quart. Values of 20 to 30 units per quart were not uncommon during the summer

months whereas values of 8 units and less were frequently observed during the winter months. These results in a general way corroborate those of other investigators.

Regarding the factors which contribute to the variability in the vitamin D content of milk, the amount of exposure of the cows to sunlight probably plays the major role. This is strikingly indicated by the excellent correlation between the vitamin D potency of the milk and the amount of available sunshine as shown in figures 1, 2 and 3. Undoubtedly even better correlation might have been obtained if a record had been kept of the hours of actual exposure to sunlight as well as of the ultraviolet intensity of the sunlight. The lack of agreement during February is to be explained on this basis.

It follows from the above that the vitamin D contained in ordinary dairy feeds, particularly roughages and silage, however important this source may be to the general well being and productiveness of the dairy cow, contributes relatively little to the vitamin D content of the milk. Furthermore the rapid drop in the antirachitic potency of milk which follows the decrease in exposure of the cows to sunlight suggests that under ordinary conditions of management and feeding the dairy cow has practically no opportunity to build up a reserve of vitamin D during lactation.

In comparing the Holstein and Guernsey samples as shown in tables 2 and 3 it is interesting to note that there was little difference in the antirachitic potency of the milk fat. However, because of the higher per cent of fat in the milk of the latter breed, the calculated vitamin D content of the milk was greater.

SUMMARY

1. A method is presented for the concentration of the antirachitic factors in milk fat thus making possible the biological assay of fats of low potency.

2. The monthly assay of milk fats from several sources over a period of 2 years shows that milk may vary as much as 900 per cent in antirachitic potency. Highest values were obtained during July, August or September and lowest usually in February. Vitamin D values ranging from 4.8 to 43.8 U.S.P. units per quart of milk were observed in the case of Guernsey milk whereas the extreme values for Holstein milk were 3.1 to 27.7 U.S.P. units per quart.

3. The close correlation between the antirachitic potency of milk and the amount of available sunshine indicates that the exposure of cows to sunlight is the major factor contributing to the vitamin D content of milk.

4. Apparently the cow has little or no opportunity to store vitamin D during lactation under ordinary dairy management conditions.

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THE RELATION BETWEEN CALCIUM RETENTION AND THE STORE OF CALCIUM IN THE BODY, WITH PARTICULAR REFERENCE TO THE DETERMINATION OF CALCIUM REQUIREMENTS [1]

B. W. FAIRBANKS AND H. H. MITCHELL

Division of Animal Nutrition, Department of Animal Husbandry, University of Illinois, Urbana

ONE FIGURE

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The requirement of protein for the growth of an animal is measured by the retention of nitrogen under conditions favoring the most rapid rate of growth. This is true because all (or practically all) of the nitrogen retained during growth becomes an integral part of the protoplasmic or structural framework of the newly developed tissues. Very little if any of the nitrogen so retained is laid down as an inert deposit subject to mobilization when the needs of the tissues for protein and other nitrogenous complexes are not adequately covered by the dietary supply. Under these conditions the nitrogen retained daily measures the day to day need for this element.

In the case of other indispensable nutritive elements, such as calcium, phosphorus and iron, the situation is quite different. With each of these elements there is provision in some of the tissues for an inert deposit subject to call, as the needs of the body dictate. In the case of calcium and phosphorus these inert deposits perform the purely mechanical

[1] This investigation was made possible by the donation of funds to the University of Illinois by the American Dry Milk Institute, Inc.

function of giving rigidity to the skeletal and cartilaginous framework of the body, but they are, nevertheless, a readily available source of supply for calcium (McCrudden, '12; Bauer, Aub and Albright, '29) and phosphorus to the soft tissues as the need arises.

With such elements the requirements for animal growth are not necessarily measured by their rate of retention in the body under conditions favoring the most rapid development of the tissues, for two reasons. In the first place, the retention may include a variable fraction used to fill up depleted stores. This fraction is not a part of the day to day requirement for the element in question, since it is the expression merely of a preceding state of inadequate nutrition with reference to it.

In the second place the retention may include a fraction used to fill to repletion the stores developing in the new tissues although repletion may not be required for maximum physiological efficiency. It is quite conceivable, with reference to calcium for example, that the skeleton of a rat would hold 65 per cent of ash, but that a content of 50 per cent is all that is needed to maintain a proper rigidity of the bones, and a normal content of calcium in the soft tissues in so far as this content is dependent upon the calcium content of the skeleton.

At the present time, the existence of the latter fraction is somewhat hypothetical though not improbable. Sherman and Booher ('31) have shown that the calcium content of rats growing on rations varying in their content of this element from 0.16 to 0.50 per cent is closely dependent upon the level of dietary calcium. Sherman and Campbell ('35) proved that a diet containing 0.2 per cent of calcium was deficient in this nutrient for rats as indicated by impairment of growth, vitality, fertility and ability to nourish the young. Unfortunately for the purposes of this discussion the carcasses and the bones of these rats were not analyzed for calcium. On the other hand it has been abundantly demonstrated (Friedman, '35) that the human infant retains the calcium from

cow's milk at a rate two to three times as rapid as that of breast milk in conformance with the much larger content of calcium in cow's milk (0.114 per cent) than in human milk (0.031 per cent). It is doubtful that any benefit is derived from this more rapid rate of bone calcification. Hence, there is room for the belief that benefits accrue to increasing rates of calcification of the skeleton only up to a point considerably short of complete saturation. If this is true for the growing animal, it may be equally true that in the adult maximum physiological efficiency is consistent with incomplete saturation of the bones with calcium. However, until the minimum percentage saturation of the calcium stores compatible with maximum physiological performance has been determined, it would seem to be the wiser course (certainly the safer course) to consider complete saturation of the stores as the ideal condition, and to include in the calcium requirement for growth (or maintenance) that amount needed to produce (or maintain) a condition of full repletion.

The highly variable retentions of calcium by children reported in the literature may conceivably be due to the variable condition of their skeletal tissues with respect to calcium saturation. This has been emphasized particularly by Daniels and her associates ('34) and by Wang, Kaucher and Frank ('28). A variable saturation of the calcium stores of the experimental subjects is probably an important factor in the discrepancies among estimates of the calcium requirements of children: For example, whether these daily requirements may be largely covered by the calcium contained in a pint of milk or less (Daniels, Hutton, Knott, Wright and Forman, '35), a quart of milk (Sherman and Hawley, '22), or more than a quart of milk (Jeans and Stearns, '32). In fact, Daniels and co-workers ('35) interpret high retentions of calcium in children as being due to previous depletion, and Clark ('26) in his studies on the calcium retentions of adults likewise correlates high retentions with previous inadequate nutrition. However, these interpretations are not based upon definite evidence of the pre-experimental state of the calcium stores

of the subjects, while, in apparent contradiction to them, Wang, Kaucher and Frank ('28) observed that their underweight subjects did not store calcium at a much more rapid rate than their vigorous normal subjects; also no correlation is evident in these experiments between the daily calcium retention per kilogram of body weight and the percentage under weight of the children.

Boldt, Brahm and Andresen ('29) have reported some experimental evidence for the belief that previous depletion of calcium stores will accelerate its storage. The calcium and phosphorus metabolism of two infants was studied over a period of 3 months, divided into three experimental periods. In the first and third periods, the infants were given human milk, and in the second period cow's milk containing a much greater content of Ca and P. The retentions of these elements were highest in the second period, but in the third period the retentions were much lower than in the first period, although the intake of calcium was somewhat greater. The results may be explained on the plausible assumption that the calcium stores were more nearly repleted at the start of the third period than at the start of the first, the greater degree of repletion inducing a lower rate of calcium deposition.

The problem of the effect of the previous condition of the calcium stores on the rate of retention of calcium during periods of experimental feeding is important to the determination of the calcium requirement of man and of all animals, and since available information appears insufficient to solve the problem, the experiment to be reported in this paper was carried out.

PLAN OF EXPERIMENT

In this experiment the calcium stores of rats were filled to different levels by subsistence in a preliminary period on diets varying in their content of calcium from 0.18 to 1.25 per cent. In a subsequent period all rats were placed upon the diet containing 1.25 per cent of calcium and their retentions of this element were then measured both by carcass analysis and by collection and analysis of urine and feces.

The paired-feeding method was used throughout these experiments in order that the comparative effects of the diets upon the calcium stores of the rats would relate solely to the calcium contents of the diets used, and not to the amounts of them consumed.

In the preliminary period three groups of rats, each consisting of eleven or twelve pairs, were used in three comparisons: First, a comparison of a ration containing 0.18 per cent of calcium with one containing 1.25 per cent; second, a comparison of a ration containing 0.32 per cent calcium with one containing 1.25 per cent; and third, a comparison of a ration containing 0.49 per cent calcium with one containing 1.25 per cent. The rats were started at weights of 40 to 60 gm. and were fed in this manner for 28 days in the case of the first two comparisons, but for only 14 days in the case of the last comparison. During this time the growth of the rats was somewhat restricted by restriction of food intake. At the termination of the preliminary period, three or four pairs of rats in each of the three series were sacrificed for chemical analysis. The results of this analysis afforded information of the calcium saturation of the carcasses effected by the various experimental diets. Compared with analyses of control rats analyzed at the initial experimental weights, estimates of the relative storages of calcium on the various diets are possible.

At the end of the preliminary period all rats were placed upon the highest level of calcium, 1.25 per cent, eight pairs in each of the three series being continued in this manner, still retaining the paired-feeding technic. On four pairs in each series, the combined excreta were collected daily and composited weekly for analysis of their calcium content. Feces markers of Fe_2O_3 or Cr_2O_3 were used at the end of each experimental week. When the rats reached approximately 200 gm. in weight, or somewhat more, they were all sacrificed for measurement and analysis. During this period, growth was not restricted and the intake of food was such that the desired final weights were reached in some pairs in only 2 to 3 weeks; in a few pairs, in 5 to 6 weeks.

The rations used all contained 23.21 per cent of dried fat-free whole egg, 10 per cent of sucrose, 10 per cent of lard, 3.43 per cent of a salt mixture[2] containing no calcium or phosphorus, 2 per cent of cod liver oil, 7 per cent of dried yeast, 39.36 per cent of cornstarch, and 5 per cent of a mixture of $BaSO_4$ and $CaHPO_4.2H_2O$, the components of which were adjusted to give the desired percentage of calcium.

The rations contained an average of 20.75 per cent of protein ($N \times 6.25$) and 4.47 calories per gram. The average contents of calcium and phosphorus were 0.185 and 0.354 per cent, respectively, for diet A, 0.318 and 0.484 per cent for diet B, 0.487 and 0.574 per cent for diet C, and 1.252 and 1.121 per cent for diet D. Calcium was determined in food, combined excreta, and carcasses by the method of McCrudden ('10); phosphorus was determined by the Pemberton-Kilgore method as modified by Hibbard ('13).

EXPERIMENTAL RESULTS

It is not necessary to give the individual body weight records of the rats during the preliminary period because they have no intimate bearing on the problem under study. In the comparison of the 0.18 and 1.25 per cent calcium levels, the rat on the higher level of calcium, but receiving the same amount of food as his pair mate, gained the faster in eight of the eleven pairs, the average excess gain being 2.08 gm., the standard deviation of differences in gain between pair mates being 3.87 gm., and the probability that this was a

[2] The mineral mixture contained:

	Per cent
Potassium citrate, $K_3C_6H_5O_7.H_2O$	20.52
Potassium sulfate, K_2SO_4	2.08
Potassium chloride, KCl	19.61
Ferric citrate, $FeC_6H_5O_7.1.5\ H_2O$	1.48
Potassium iodide, KI	0.0047
Manganese sulfate, $MnSO_4$	0.0184
Sodium fluoride, NaF	0.0578
Potassium aluminum sulfate, $K_2Al_2(SO_4)_4$	0.0057
Sodium chloride, NaCl	9.07
Magnesium chloride, $MgCl_2.6H_2O$	47.17

purely fortuitous outcome being 0.059 according to the method of analysis of Student ('08). This difference in gain is slight but on the borderline of statistical significance. In the other comparisons the differences in gain between pair mates were quite insignificant, the pairs dividing five to six in favor of the low calcium level in the comparison of the 0.32 and 1.25 per cent calcium rations, and five to seven in favor of the lower level in the comparison of the 0.49 and 1.25 per cent calcium rations. The daily gains in body weight in the three comparisons averaged 2.33, 2.81, and 5.07 gm., respectively.

The results of the analysis for calcium of the carcasses of the rats killed at the termination of the preliminary period are summarized in table 1. The average percentages of calcium (computed on the empty weight) in the first comparison were 0.698 for the rats on the ration containing 0.18 per cent of calcium and 0.895 for the rats on the ration containing 1.25 per cent of calcium. In the second comparison, the percentages were 0.704 for the rats on the ration containing 0.32 per cent of calcium and 0.820 for the rats on the ration with a calcium content of 1.25 per cent. In the last comparison the average percentages were 0.682 and 0.733, respectively, for the rations containing 0.49 and 1.25 per cent of calcium.

Although the numbers of paired analyses in each comparison were small, the differences between pair mates were quite definitely significant statistically in all cases, as the computations of table 2 indicate. The probabilities given in the last column of the table are so small that they may be neglected. Consequently it may be concluded rationally that the observed differences in the calcium content of paired rats were produced by the different levels of calcium in the diets upon which the rats had subsisted. In other words, the different levels of calcium in the experimental diets had in fact induced different degrees of saturation of the calcium stores. Hence, the purpose of the preliminary period of feeding was realized.

It will be noted that there was considerable variation among the calcium contents of rats reared upon the same experimental diets. This is particularly noticeable with the rats

TABLE 1

Calcium content of selected rats at termination of preliminary period

PAIR NUMBER	PER CENT CA IN RATION	BODY WEIGHT		AVERAGE DAILY GAIN IN BODY WEIGHT	PER CENT CA IN CARCASS[1]
		Alive	Empty		
		gm.	*gm.*	*gm.*	
1	0.18	123	118	2.3	0.66
	1.25	124	123	2.5	0.82
3	0.18	102	98	2.1	0.70
	1.25	105	103	2.4	0.98
11	0.18	100	97	1.8	0.73
	1.25	105	101	2.0	0.88
2	0.32	140	136	3.4	0.58
	1.25	139	137	3.2	0.70
5	0.32	116	114	2.5	0.74
	1.25	115	113	2.5	0.85
11	0.32	98	97	1.8	0.80
	1.25	108	106	2.1	0.91
1	0.49	103	101	4.3	0.67
	1.25	103	102	4.2	0.69
2	0.49	94	93	3.9	0.70
	1.25	97	95	3.8	0.72
3	0.49	138	137	6.0	0.69
	1.25	137	137	6.0	0.76
10	0.49	137	136	5.1	0.67
	1.25	139	138	5.3	0.76

[1] Computed on the empty weight.

TABLE 2

Statistical analysis of the calcium percentages summarized in table 1

CALCIUM LEVELS COMPARED	NUMBER OF PAIRS OF RATS	DIFFERENCES IN CALCIUM CONTENT BETWEEN PAIR MATES		PROBABILITY OF A FORTUITOUS OUTCOME
		Mean[1]	Standard deviation	
0.18 vs. 1.25	3	+ 0.197	0.059	0.021
0.32 vs. 1.25	3	+ 0.116	0.0065	$<$ 0.014
0.49 vs. 1.25	4	+ 0.051	0.031	0.034

[1] The positive sign indicates that the rat on the higher calcium ration possessed on the average the higher content of calcium.

reared upon the diet containing 1.25 per cent of calcium, which was used in all three comparisons. In particular, the values obtained in the third comparison are quite uniformly low. The rates of gain in body weight of the rats in the preliminary period, presented in the fifth column of table 1, help to explain the variations in calcium content of the carcasses. There is a very evident negative correlation between rate of growth and calcium content of body. More evidence of this correlation, as well as a discussion of its significance, will be given later.

The initial calcium content of the rats may be estimated from analyses of eighteen control rats, eleven males and seven females, ranging in weight from 41 to 62 gm. and averaging 50 gm. These rats were representatives of the same litters from which the experimental rats were selected. Their average intestinal fill was 5.55 per cent and their average calcium content on the empty weight basis was 0.801 per cent. The coefficient of variation of the eighteen analyses was 7.5 per cent. No sex difference in body calcium was evident.

The calcium content of the rats at the end of the preliminary feeding period may be estimated for each group of rats reared on the same ration from the average analyses of the rats killed at that time. For the latter rats themselves, the analyses actually obtained were used. The estimated difference between final and initial calcium contents represents the calcium stores in the body, the amount of which may be compared with the calcium intake. The averages of such estimations and observations for the three ration comparisons will be found in table 3.

The ration containing 0.18 per cent of calcium induced a storage of this element averaging only 62 per cent of that effected by the ration containing 1.25 per cent of calcium. The rations containing 0.32 and 0.49 per cent of calcium permitted retentions of 76 and 87 per cent, respectively, of those brought about by the ration containing 1.25 per cent of calcium. However, the calcium intake on the lowest level of calcium was completely utilized in growth, while those of the

higher levels were utilized to the extent of 76, 55 and 25 per cent, in order. The complete utilization of the calcium on the lowest calcium ration, as well as the constant utilization with variable rates of growth on the highest calcium ration, averaging 2.38, 2.83 and 5.08 gm. per day in the three comparisons, confirm the results of Ellis and Mitchell ('33) and testify to the reality of their theory that "in the growing rat, and possibly in all growing animals, there is no integral requirement of calcium for maintenance."

The preliminary feeding evidently induced different degrees of saturation of the calcium stores, standing in relation to

TABLE 3

Summary of results of preliminary feeding period

PER CENT CA IN DIET	NUMBER OF RATS	LENGTH OF FEEDING PERIOD IN DAYS	AVERAGE GAIN IN BODY WEIGHT	AVERAGE FOOD INTAKE	AVERAGE INTAKE OF CALCIUM	AVERAGE CA RETENTION	
						In milli-grams	In per cent
			gm.	*gm.*	*mg.*		
0.18	11	28	64	175	336	356	106
1.25	11	28	67	175	2336	578	25
0.32	11	28	78	192	610	465	76
1.25	11	28	79	192	2559	615	24
0.49	12	14	78	153	746	412	55
1.25	12	14	77	153	1916	476	25

one another as 62, 76, 87 and 100. The purpose of the main feeding period was to observe whether these differences in calcium saturation would induce differences in calcium retention when all rats were placed upon the ration containing the highest percentage of calcium, i.e., 1.25. The growth data of this period of uniform feeding are summarized in table 4.

No significant differences in growth were observed between pair mates except in the first comparison, in which the preliminary rations contained 0.18 and 1.25 per cent of calcium. In this comparison, in seven of the eight pairs the rat prefed on the higher calcium level grew the faster, when measured both by body weight gain and by final body length, measured from tip of nose to root of tail. The average increment in

TABLE 4

Body lengths and weights, gains in weight and food consumption for the experimental period. All rats on same ration containing 1.25 per cent Ca. All weights expressed in grams

PRELIMINARY RATION	PAIR 1		PAIR 2		PAIR 3		PAIR 4		PAIR 5		PAIR 6		PAIR 7		PAIR 8		AVERAGES	
	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca
Preliminary rations	0.18 per cent Ca vs. 1.25 per cent Ca																	
Rat number and sex	3m	4m	7m	8m	9m	10m	11m	12m	13f	14f	15f	16f	17f	18f	19f	20f	...	...
Initial body weight	129	132	120	117	118	114	108	111	104	104	97	102	94	97	95	94	108	109
Final body weight	224	229	214	223	215	228	209	228	199	220	179	191	190	185	186	189	202	212
Total gain	95	97	94	106	97	114	101	117	95	116	82	89	96	88	91	95	94	103
Body length, mm.[1]	202	211	206	209	208	209	202	210	203	216	203	200	196	201	198	200	202	207
Total food	230	230	235	234	258	258	267	267	360	361	420	420	414	414	455	455	330	330
Length of test in days	16	16	18	18	18	18	17	17	28	28	41	41	36	36	40	40	27	27
Preliminary rations	0.32 per cent Ca vs. 1.25 per cent Ca																	
Rat number and sex	25m	26m	29m	30m	31f	32f	35f	36f	37m	38m	39f	40f	41m	42m	43m	44m	...	...
Initial body weight	135	133	135	134	126	126	114	118	133	136	109	111	123	122	127	126	125	126
Final body weight	229	222	232	230	199	204	183	179	230	238	184	188	219	225	226	239	213	216
Total gain	94	89	97	96	73	78	69	61	97	102	75	77	96	103	99	113	88	90
Body length, mm.[1]	213	210	212	212	203	208	202	187	202	207	212	212	208	209	211	219	208	208
Total food	234	234	309	309	428	425	417	416	255	255	420	420	311	311	333	333	338	338
Length of test in days	14	14	21	21	38	38	42	42	17	17	40	40	21	21	22	22	27	27
Preliminary rations	0.49 per cent Ca vs. 1.25 per cent Ca																	
Rat number and sex	55m	56m	57m	58m	59m	60m	61m	62m	63m	64m	65m	66m	69m	70m	71m	72m	...	...
Initial body weight	138	138	131	128	131	127	130	128	117	120	114	115	140	140	121	124	128	127
Final body weight	236	243	228	232	233	230	226	210	200	203	182	201	238	239	214	220	220	222
Total gain	98	105	97	104	102	103	96	82	83	83	68	86	98	99	93	96	92	95
Body length, mm.[1]	217	212	215	207	211	215	210	211	207	210	200	206	213	210	210	208	210	210
Total food	275	276	289	289	274	274	292	292	338	338	290	290	295	294	309	309	295	295
Length of test in days	18	18	19	19	17	17	21	21	31	31	26	26	16	16	22	22	21	21

[1] Measured from anus to tip of nose.

gain was 9.62 gm., and in body length, 4.75 mm.; the standard deviations of differences in gain between pair mates was 8.02 gm., and in body length, 4.76 mm. The probabilities of a fortuitous outcome are so small, 0.0078 and 0.017, that they may be neglected. It may be concluded, therefore, that subsistence upon a ration containing as low as 0.18 per cent of calcium definitely impairs growth, not only simultaneous with its consumption, but also in a subsequent period of adequate calcium nutrition. No evidence of this sort was obtained for levels of 0.32 and 0.49 per cent of calcium.

The calcium contents of the carcasses of the rats, with estimates of calcium storage involving estimates of initial calcium content based upon the analysis of rats on the same preliminary rations made at the end of the preliminary feeding period (table 1), are presented in table 5. The calcium contents of the various pair mates with different preliminary treatment are not to be distinguished from one another statistically in any of the three comparisons. Evidently the inequalities in initial calcium content induced by different levels of calcium feeding were removed during this period of uniform feeding. In agreement with this finding, the estimated calcium retentions average higher for the rats reared on the lower calcium diets in all comparisons, the average percentage increments being 19.7, 3.1 and 13.0, respectively; in milligrams, these increments average 137, 24 and 99, respectively. The standard deviations of differences in calcium storage between pair mates were 110, 124 and 164 mg. in the three comparison groups, while the probabilities of a chance outcome are 0.0066, 0.31 and 0.078, respectively. Only the first of these probabilities is small enough to neglect, so that only in the first comparison of rats pre-fed on 0.18 and 1.25 per cent levels of calcium may it be said with certainty that an initial lower saturation of the calcium stores promotes a more rapid storage of calcium in a subsequent period of uniform feeding. However, the inconclusiveness of the data in the other comparisons may have been the result of the inaccuracy in the method of estimation of the initial calcium contents of the rats, for reasons that will be developed later.

TABLE 5

Calcium contents of rats at end of experimental period with estimates of calcium storages

PRELIMINARY RATION	PAIR 1		PAIR 2		PAIR 3		PAIR 4		PAIR 5		PAIR 6		PAIR 7		PAIR 8		AVERAGES	
	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca
Preliminary rations	0.18 per cent calcium vs. 1.25 per cent calcium																	
Rat number and sex	3m	4m	7m	8m	9m	10m	11m	12m	13f	14f	15f	16f	17f	18f	19f	20f	...	...
Ca in carcass, per cent, obs.[1]	0.66	0.70	0.72	0.65	0.76	0.67	0.55	0.60	0.88	0.81	0.94	0.97	0.84	0.98	0.87	0.90	0.778	0.785
Ca in carcass, mg., obs.	1494	1610	1529	1437	1640	1523	1131	1365	1759	1770	1679	1826	1589	1776	1611	1710	1554	1627
Initial Ca content, mg., est.[2]	866	1137	803	1002	789	976	719	949	691	886	642	868	621	823	628	797	720	930
Ca stored, mg., est.	628	473	726	435	851	547	412	416	1068	884	1037	958	968	953	983	913	834	697
Preliminary rations	0.32 per cent calcium vs. 1.25 per cent calcium																	
Rat number and sex	25m	26m	29m	30m	31f	32f	35f	36f	37m	38m	39f	40f	41m	42m	43m	44m	...	...
Ca in carcass, per cent, obs.[1]	0.55	0.69	0.68	0.83	0.95	0.99	1.01	1.03	0.66	0.71	1.00	0.95	0.79	0.79	0.73	0.68	0.796	0.834
Ca in carcass, mg., obs.	1261	1548	1566	1894	1883	2006	1827	1842	1520	1685	1840	1799	1722	1790	1658	1677	1660	1780
Initial Ca content, mg., est.[2]	922	1058	922	1066	859	1000	774	935	908	1082	739	877	838	968	866	1000	854	998
Ca stored, mg., est.	339	490	644	828	1024	1006	1053	907	612	603	1101	922	884	822	792	677	806	782
Preliminary rations	0.49 per cent calcium vs. 1.25 per cent calcium																	
Rat number and sex	55m	56m	57m	58m	59m	60m	61m	62m	63m	64m	65m	66m	69m	70m	71m	72m	...	...
Ca in carcass, per cent, obs.[1]	0.71	0.70	0.73	0.69	0.65	0.70	0.70	0.75	0.90	0.88	0.91	0.82	0.64	0.69	0.95	0.74	0.774	0.746
Ca in carcass, mg., obs.	1730	1713	1672	1633	1534	1624	1582	1583	1801	1778	1632	1630	1536	1644	2040	1612	1691	1652
Initial Ca content, mg., est.[2]	900	968	853	894	853	887	846	894	757	836	737	799	914	982	784	865	831	891
Ca stored, mg., est.	830	745	819	739	681	737	736	689	1044	942	895	831	622	662	1256	747	860	761

[1] Obs. = observed. [2] Est. = estimated.

Fortunately, other data on the calcium retentions of the rats are available from the calcium metabolism experiments undertaken with four pairs of rats in each of the three comparisons. These data are summarized in table 6. In all of

TABLE 6

Calcium metabolism data for experimental period

RAT NO.	CALCIUM CONTENT OF PRELIMINARY DIET	EXPERIMENTAL PERIOD	CALCIUM INTAKE	CALCIUM EXCRETED	CALCIUM BALANCE
	per cent	*days*	*mg.*	*mg.*	*mg.*
3	0.18	16	2878	2102	776
4	1.25	16	2878	2313	565
7	0.18	18	2942	2160	782
8	1.25	18	2935	2244	691
9	0.18	18	3230	2340	890
10	1.25	18	3224	2498	726
11	0.18	17	3341	2580	761
12	1.25	17	3341	2594	747
25	0.32	14	2925	2255	670
26	1.25	14	2925	2416	509
29	0.32	21	3702	2996	706
30	1.25	21	3702	2964	738
31	0.32	38	4825	3816	1009
32	1.25	38	4799	3872	927
35	0.32	42	4666	3724	942
36	1.25	42	4660	4000	660
55	0.49	18	2961	2033	928
56	1.25	18	2967	2198	769
57	0.49	19	3113	2297	816
58	1.25	19	3113	2361	752
59	0.49	17	2979	2302	677
60	1.25	17	2973	2346	627
61	0.49	21	3135	2352	783
62	1.25	21	3135	2463	672

the twelve pairs of rats but one, the rat pre-fed upon the lower level of calcium retained the greater amount of calcium in the period of uniform feeding, the excess storage averaging 120, 123 and 96 mg., respectively, for the three comparison groups. The standard deviations of differences between pair mates are, in order, 75, 114 and 43 mg., and the probabilities of a fortuitous outcome, 0.035, 0.079 and 0.015. The second probability is still too large to neglect, but the whole picture is a clear demonstration of the fact that differences in the degree of saturation of the calcium stores will produce inequalities in calcium retention in a period of uniform high-calcium feeding, such that the lower the initial saturation, the more rapid the subsequent rate of retention. Throwing all of the pairs together into one statistical analysis, since the average pair differences are not statistically distinct, gives a mean difference of 113 mg., a standard deviation of 84 mg., and a probability (Student, '25) of a chance outcome of only 0.0005.

The estimations of phosphorus retentions based upon carcass analyses are irregular and offer no evidence that the previous condition of calcium (and probably phosphorus) saturation has modified the subsequent rate of phosphorus storage. However, the phosphorus balance data from the four pairs of rats in each comparison studied in this manner, suggest, in conformance with the calcium data of table 6, that the rate of phosphorus retention has been modified by the nature of the preliminary feeding. The average increments in phosphorus retention of the rats pre-fed on the high-phosphorus ration over their pair mates pre-fed on the low-phosphorus ration are, for the three comparisons, 51, 129 and 102 mg., respectively. However, none of these averages is statistically significant, the probability that chance alone could account for them being, respectively, 0.18, 0.09 and 0.10. The average ratio of retained calcium to retained phosphorus was 2.12 to 1, with no clear effect of the type of pre-feeding.

The calcium and phosphorus contents of the forty-eight rats surviving to the end of the experiment were not appreciably

affected by the different methods of pre-feeding. Hence a number of correlation studies were undertaken to throw light upon the factors modifying the content of the body in calcium and phosphorus. The product-moment correlation method of Pearson was used throughout. The correlation coefficients were calculated from the unclassified original data.

We were at first disturbed by the fact that the percentages of calcium in these rats were much lower than those reported by Sherman and Booher ('31) for rats of the same body weight on high calcium levels, the latter ranging from 1 per cent, or only slightly less, to 1.2 per cent. Our values averaged 0.785 per cent, and ranged from 0.55 to 1.03. However, our experimental rats grew at a very rapid rate, averaging 4.3 gm. daily and attaining values up to 6.7 gm. daily in individual rats. This was a much more rapid growth than that of the rats of Sherman and Booher, a fact that suggested a study of the relationship between rate of growth, as measured by average daily gain in body weight, and the calcium content of the body. Figure 1 illustrates this relationship for the forty-eight rats of the three comparisons in this experiment. The relationship is evidently a close one, the correlation coefficient being -0.929 ± 0.013, the sign meaning that the more rapid the growth, the lower the calcium content of the body. The rate of gain in weight was largely determined by the daily intake of food, the correlation coefficient for these two variables being $+0.910 \pm 0.017$.

There are two possible explanations of the inverse relationship between rate of growth and the calcium content of the body: First, that there is an inverse relationship between the rate of growth and the rate of calcification of the skeleton, and, second, that there is an inverse relationship between the rate of growth and the percentage of skeletal tissue in the body. In favor of the first explanation, Outhouse and Mendel ('33) have shown clearly that more rapid growth for rats of the same weight is associated with bones containing more water and less mineral matter. To test the second explanation the calcium contents of the carcasses of the forty-eight

rats were correlated with the ratios of calcium to phosphorus in the carcasses, which averaged 1.37 to 1. The correlation coefficient in this case is $+ 0.584 \pm 0.064$. Since this correlation is significantly positive, we may conclude that as the rate of growth increases, in response to an increasing consumption of food, the proportion of soft tissues in the body increases; conversely, a retardation of growth affects the soft tissues of the body more than the skeletal tissues. This conclusion

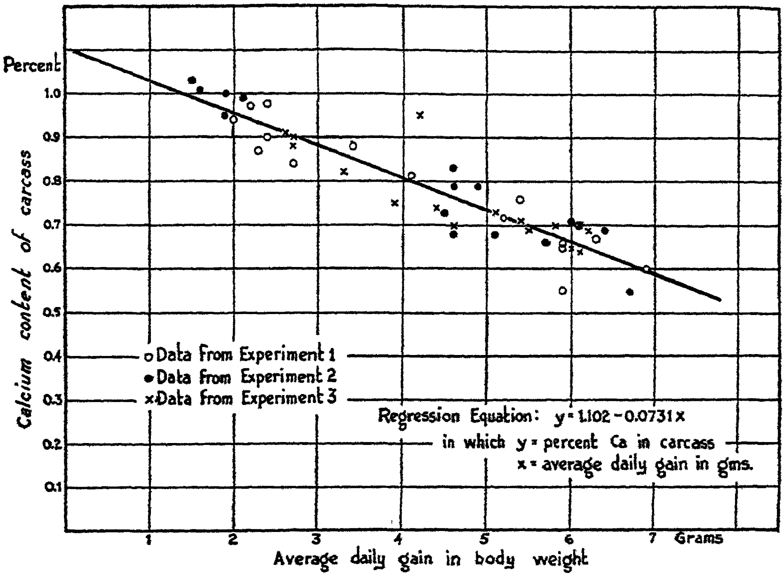

Fig. 1 Relation between the calcium contents of the bodies of the experimental rats and their average rates of growth for the previous several weeks.

is in agreement with the frequently demonstrated fact that skeletal growth is less easily retarded than the growth of the soft tissues; it is also less readily accelerated.

The calcium to phophorus ratios in the carcasses of the experimental rats ranged from 0.95 to 1 for a rat that gained an average of 6.7 gm. daily to 1.65 to 1 for a rat that gained only half as fast, the average ratio being 1.37 to 1. Assuming that 99 per cent of the body calcium is located in the skeleton (Bessey, King, Quinn and Sherman, '35) and that the ratio

of calcium to phosphorus in the skeleton is 1.94 to 1, it may be estimated that from 48 to 84 per cent of the body phosphorus, with an average of 70 per cent, was located in the bones in these experimental rats. This wide range in the proportion of skeletal phosphorus is indicative of the marked influence of the amount of food consumed on the ratio of soft tissues to bones in the body and necessarily on the proportions existing among the nutrients retained during growth.

The frequently noted greater calcium content of the bodies of female than of male rats of like age, or similar difference with respect to calcification of the bones (Hammett, '25), appears to be entirely the result of the slower growth of the female. When the correlation between calcium content of carcass and rate of growth is considered, no sex differences appear. If the regression equation given in figure 1 is used to estimate the calcium content of each of the forty-eight experimental rats from its average daily gain in body weight, the algebraic mean of the deviations of the estimates from the observed values is + 0.0024 per cent for the thirty-four male rats, and — 0.0050 for the fourteen female rats. The average errors of estimate, disregarding signs, are 0.038 for the males and 0.032 for the females. No significant difference appears to exist between the accuracies of estimation for the two sexes. If the sex difference commonly accepted actually existed, one might expect, not only a significant and considerable tendency to under-estimate the calcium content of female rats by the use of a regression equation based on male and female data, but also a greater mean error of estimate (disregarding signs) for the female rats, because the regression equation is derived from the data obtained from a group of rats consisting of thirty-four males and only fourteen females.

DISCUSSION

The bearing of these experimental findings on calcium metabolism studies, particularly those concerned with the determination of the calcium requirements of growing animals, seems clear. Since the extent to which the skeleton is saturated with respect to calcium is a determinant in the rate of calcium retention on a diet adequate in its content of this element (and presumably on any diet), calcium retentions observed under conditions of adequate nutrition are not measures of calcium requirements unless, through proper preliminary treatment, the experimental subjects have been saturated with respect to their calcium stores. Otherwise, the calcium retentions observed will exceed the actual requirements by the amount used to replete these stores, the condition of which is an expression simply of previous inadequate nutrition. Furthermore, in the evaluation of experiments in which this precaution has not been taken, more significance can reasonably be attached to the lower rates of calcium retention than to the higher as measures of day to day requirements.

In the above discussion complete saturation of the calcium stores is assumed to be the ideal initial condition of experimental subjects, and the maintenance of this condition during growth the measure of adequate calcium nutrition. However, wholly adequate calcium nutrition may be compatible with a condition of the skeleton short of complete calcium saturation. Hence, a significant problem in calcium metabolism is the determination of the lowest degree of calcium saturation compatible with maximum physiological efficiency. Conceivably this degree of saturation will be affected by the prevailing physiological condition and hence would vary with animals of different age, sex, etc.

Since the calcium content of the body and of the bones is markedly affected by the amount of food consumed as well as by its content of calcium, the control of food intake in calcium metabolism studies is essential to the most significant results.

CONCLUSIONS

1. The calcium content of growing rats is dependent upon at least two factors: a) the calcium content of the diet if the diet contains a percentage of calcium inadequate for maximum storage, and b) the rate of growth, dependent in turn largely upon the rate of food consumption. The former relation is a direct one, the latter is inverse, rapid gains being associated with low calcium contents.

2. The inverse relation between rate of growth and calcium content of body is the result of two tendencies: a) the tendency for rapid growth to be associated with slow calcification of the bones, and b) the tendency for rapid growth to be associated with high ratios of soft tissue to skeletal tissue. The growth of skeletal tissue is much more difficult to modify by feeding, either in the direction of retardation or of acceleration, than is the growth of the soft tissues.

3. The commonly accepted belief that females, as compared with males of the same age, exhibit greater percentages of calcium in their bodies and in their bones seems to be entirely referable to their slower growth.

4. Very low levels of calcium in the diet may definitely retard growth aside from their effect upon appetite, not only during the consumption of such diets, but also in a subsequent period of adequate calcium nutrition.

5. There appears to be no requirement of calcium for maintenance in the growing animal.

6. Differences in the degree of saturation of the skeletal tissues with respect to calcium, brought about by previous subsistence upon diets differing in their contents of this element, produce inequalities in the retention of calcium under uniform conditions of calcium nutrition, such that low saturation is associated with subsequent high retention of calcium.

7. It follows that the rate of calcium retention by growing animals under conditions of adequate nutrition measures the requirement of calcium only when the calcium stores have been saturated by appropriate pre-feeding. Otherwise the observed calcium retentions will be greater than the day to day requirements of calcium.

8. Since the calcium content of growing rats (and presumably of other animals) and the extent of calcification of their bones, is dependent upon the amount of food consumed, as well as upon its mineral content, the control of the food intake of experimental animals in calcium nutrition studies is essential to the greatest accuracy of all comparisons made and to the greatest significance of the conclusions deduced.

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A STUDY OF THE DIETARY FACTORS CONCERNED IN NUTRITIONAL MUSCULAR DYSTROPHY[1]

SERGIUS MORGULIS AND HOWARD C. SPENCER
Department of Biochemistry, University of Nebraska, College of Medicine, Omaha

(Received for publication February 17, 1936)

I. CAUSATION AND PREVENTION OF NUTRITIONAL MUSCULAR DYSTROPHY

The experiments of Goettsch, Pappenheimer and their collaborators (Goettsch and Brown, '32; Goettsch and Pappenheimer, '31; Pappenheimer and Goettsch, '31, '34 a, '34 b; Rogers, Pappenheimer and Goettsch, '31; Victor, '34) showed, for the first time, that muscular dystrophy could be produced in laboratory animals (guinea pig, rabbit and duckling) by nutritional means. The similarity between the histological and clinical pictures obtained with these animals and those found in cases of progressive muscular dystrophy suggested that the experimental condition in animals might be closely related to the clinical condition found in man. In view of this possible relationship it was deemed worthwhile to investigate more closely rabbits affected with nutritional muscular dystrophy, and a series of experiments were planned with this in mind.

The control diet

Our control animals were maintained on a standard diet[2] which furnished an adequate supply of vitamins A, B, E and G, and a small amount of D. The rabbits on this diet invariably made a steady gain in weight until maturity was

[1] The data presented in this paper are taken from the dissertation submitted by Howard C. Spencer to the Graduate College of the University of Nebraska, January, 1936, in partial fulfillment of the requirements for the degree of doctor of philosophy.

[2] Rabbit chow obtained from the Ralston Purina Company.

reached, appeared to be in excellent physical condition, and served as controls for the animals on the experimental diets.

Experiments with original dystrophy producing diet 13

At the outset of our experiments it was deemed advisable to produce dystrophy in rabbits by means of the diet used originally by Goettsch and Pappenheimer ('31), their diet 13, to establish definite criteria by which the manifestations of dystrophy could be recognized in the experimental animals. Furthermore, since this diet was to be used as the basic ration in the study of the factors involved in the production of dystrophy, it was of paramount importance to have a record of the behavior of rabbits on diet 13.

This diet was made by mixing 355 parts rolled oats,[3] 180 parts wheat bran,[4] 75 parts casein, 80 parts lard, 10 parts cod liver oil, 10 parts NaCl, and 15 parts $CaCO_3$. To one such batch a mixture of 50 cc. 20 per cent $FeCl_3$ and 100 cc. ether was added in a closed vessel and allowed to stand, with frequent shaking, for about $\frac{1}{2}$ hour. The food was then spread out in shallow trays and left over night to allow the ether to evaporate. To this was now added 275 parts of skim milk powder and mixed thoroughly in a mechanical mixer. The food was prepared fresh at least once a week. Since rabbits are resistant to scurvy, orange juice was not administered.

A number of animals were maintained on this diet 13 and all became dystrophic. The average time required by the rabbits (about a month old and weighing about 500 gm.) to attain maximum weight was 26 days, while the average number of days on diet 13 before death or at any rate before a moribund condition developed was 34 days (28 to 43 days). This corresponds fairly well with the range of 14 to 50 days, which Goettsch and Pappenheimer ('31) found necessary to produce definite dystrophy on this diet. Furthermore, their description of the appearance and behavior of the dystrophic rabbits agrees well with our own observations. Histological

[3] Quaker brand.

[4] Pillsbury.

examination of the dystrophic muscles revealed essentially the conditions described by Goettsch and Pappenheimer ('31). The clinical observation of the rabbit and, whenever possible, histological examination of the muscle were the means used to ascertain the extent of dystrophy. When the progress of the disease or of the recovery was followed histologically, biopsy material was removed aseptically from the gluteus maximus under novocaine anesthesia.

Experiments on the influence of ferric chloride

Since the most essential feature in the preparation of diet 13 is the treatment with the ethereal $FeCl_3$, it seemed desirable to investigate the effect of this factor upon the dystrophy producing property of the food. For this purpose experiments were made with three different diets:

a. Diet 11 (untreated diet 13).
b. Diet 207 (diet 13 treated with aqueous $FeCl_3$).
c. Diet 201 (control diet treated with ethereal-$FeCl_3$).

a. Diet 11 is prepared exactly like the diet 13 with the omission of the treatment with ethereal ferric chloride and, therefore, feeding this diet should give some indication of the role of ferric chloride in the production of dystrophy. Furthermore, since the ethereal-ferric chloride treatment was originally designed to destroy vitamin E (Waddell and Steenbock, '28), its omission makes diet 11 a fairly good source of this vitamin, and feeding experiments with it should throw light on the effectiveness of vitamin E in preventing dystrophy.

Goettsch and Pappenheimer ('31) report that rabbits react to this diet 11 exactly as they do to diet 13. Victor ('34), in a later publication, cites cases of rabbits becoming dystrophic on diet 11 as early as the twenty-third day and as late as the three hundred and thirty-first day. In our experiments, three rabbits maintained on this diet showed no signs of dystrophy at the end of a period of 69 to 83 days, which does not bear out the conclusion of Goettsch and Pappenheimer that both diet 11 and diet 13 are equally dystrophic

in their effect. Owing to lack of time, these three rabbits were shifted to diet 13, and in every case dystrophic manifestations, confirmed by histological examination, set in very soon afterward. These results indicate that diet 11 at least induces a latent dystrophic condition or in some way prepares the organism for the rapid break down which comes with the change to diet 13.

A fourth rabbit was maintained on diet 11 for 210 days without showing the slightest signs of the disease. A biopsy performed on a fifth rabbit after 121 days on diet 11 (weight 2840 gm.) showed that the muscles were entirely normal, but a second biopsy taken at the end of 212 days showed definite lesions in the muscles. In all probability the dystrophic condition developed about the one hundred and forty-seventh day, when there was a break in the body weight. However, since this animal lived 210 days on diet 11, it must have been suffering from a very mild or chronic form of dystrophy.

In contrast to this, a sixth rabbit became definitely dystrophic in 42 days on diet 11, and its condition was corroborated histologically.

In general, our results agree with those of Victor ('34), showing great variability in the time of onset, in the severity and the progress of the disease in animals maintained on diet 11. But in spite of these wide variations it can be stated that neither the presence of the ferric chloride itself in diet 13 nor the absence of vitamin E from this diet can be regarded as the principal factor in the production of nutritional muscle dystrophy.

b. Waddell and Steenbock ('28) have shown that ethereal-ferric chloride treatment destroys vitamin E in synthetic diets, while an aqueous solution of $FeCl_3$ fails to do so. For this reason we decided to compare the effect of the regular diet 13 with that of a diet treated with aqueous-ferric chloride. Our diet 207 is therefore like diet 13 except for the fact that it had been treated with aqueous-ferric chloride. The use of a water solution, of course, necessitates a longer time for evaporation. The diet tends to sour more quickly than diet 13 and was made up fresh at more frequent intervals.

The animals on this diet 207 behaved exactly like those on diet 13. Apparently the water solution of $FeCl_3$ is as effective as the ether solution in rendering the diet dystrophy producing. This bears out our conclusion from the experiments with diet 11 that the destruction of vitamin E cannot be the principal factor in diet 13 for its dystrophy producing action.

c. We studied the influence of the ethereal-ferric chloride treatment further by treating the control diet in the same manner as we treated diet 13. This diet 201 contained the same quantity of $FeCl_3$ as the original diet 13, and since the treatment destroyed the vitamin E and much of the vitamin A content of this food, it seemed that feeding it to rabbits would help to ascertain the effect of the $FeCl_3$ per se as well as that of a diminished vitamin E (and vitamin A) supply. We fed this diet 201 also supplemented with potent sources of vitamin E or vitamin A, as well as of a combination of these vitamins.

Wheat germ oil was used as a source of vitamin E, and throughout this report E will be used to designate wheat germ oil. This was a cold pressed oil, 2 cc. of which were equivalent to 100 gm. of wheat germ. The animals receiving vitamin E were given 10 to 12 drops of this oil daily by pipette. This level of wheat germ oil is somewhat higher than that used by Goettsch and Pappenheimer ('31).

An oil solution of carotene was used as a source of vitamin A, and this had a biological potency of not less than 7500 new U. S. P. units of vitamin A per gram. Five drops of this solution were administered daily to the rabbits receiving the vitamin A supplement. In this report A will always refer to this preparation. The vitamin preparations were kept in well-stoppered bottles in the ice box.

Six animals were maintained on this diet 201 for at least 200 days and none of them showed any dystrophic manifestations. Some of the rabbits (two) did not grow so well as those fed the untreated control diet, but the poorer growth may have been due to respiratory infections from which these animals suffered.

The results with diet 201 indicate that the presence of $FeCl_3$ by itself does not render a diet unsuitable for rabbits. Furthermore, the ethereal-ferric chloride treatment apparently does not destroy the principal factors which prevent the onset of dystrophy.

From the experiments on feeding diets 11, 207 and 201 we may conclude that: 1) Nutritional muscle dystrophy is not caused by the ferric chloride present in the diet. 2) The destruction of vitamin E is not the principal cause in making the diet dystrophy producing.

Substitution of a vegetable oil in diet 13

Nutritional encephalomalacia is produced in the chick by a synthetic diet similar to diet 11. Pappenheimer and Goettsch ('34 b) found that the substitution of certain vegetable oils for the lard in the original diet provides adequate protection against this disorder. We thought it advisable to test the effect of modifying diet 13 by the substitution of a vegetable oil[5] for the lard, but our rabbits became dystrophic on this modified food as quickly, if not more quickly, than on diet 13.

Diet 13 supplemented with dry alfalfa and alfalfa extracts (diets 220 to 252)

Simultaneously with the feeding experiments described above we carried out curative experiments. One of the first definite cures was effected by feeding fresh green alfalfa together with diet 13. The work with curative diets will be the subject of the next section, but we shall discuss here some of the experiments with alfalfa because they focused our attention upon this as a possible source of the factors active in preventing dystrophy. We investigated the effectiveness of dry alfalfa and of extracts prepared from both fresh and dry alfalfa in preventing nutritional muscle dystrophy. For the extracts, we used a kilogram of finely ground fresh, green alfalfa or dry alfalfa. This ground material was pressed,

[5] Mazola.

extracted with water by percolation, again pressed and re-extracted. The total aqueous extract was made up so that 3 liters were equivalent to 1 kg. of the original alfalfa. The extracts were preserved in the ice box.

The residue after the two extractions with water and with alcohol was dried and kept in a closed container.

The extracts of alfalfa did not afford any protection against the development of muscle dystrophy in rabbits fed on diet 13. Since the dry alfalfa itself is not protective, the extracts of dry alfalfa would not be expected to exert any influence either. But the fresh, green alfalfa is definitely a protective agent, and it might be expected that one or the other of the extracts, or their combination, would also prove protective. Our method of extraction did not exclude ferment action, and it is not impossible that the necessary factors might have been destroyed during the extraction. The effect of the ferric chloride treatment and the fact that dry, well-cured alfalfa is lacking in protective action suggest that the protective factor or factors are probably easily destroyed.

. On diet 220, which is diet 13 supplemented with dry alfalfa, all the rabbits became dystrophic, though the onset of the disease was slower than on diet 13 alone. The earliest onset was after 55 days on this diet and some were still normal even after 100 days.

We are led to conclude that the factor or factors present in green alfalfa, which can prevent the onset of dystrophy, is destroyed by the curing process as well as by the extraction methods employed by us, and that it is an unstable substance.

Experiments with diet 13 and lettuce

In our attempt to determine the effectiveness of green foodstuffs in preventing dystrophy we performed experiments feeding diet 13 together with fresh, green lettuce. The lettuce was selected for this purpose because it could be procured fresh daily and because it has a high vitamin E content. We were careful to select only the fresh, green leaves for feeding, a total of 50 gm. per day being fed to the rabbits in two portions during the day.

All the rabbits maintained on diet 13 supplemented daily with 50 gm. of lettuce became dystrophic, the time of onset of the disease varying from 30 to 100 days, or 53 days on the average. The feeding of lettuce has thus prolonged the period during which a rabbit could be maintained on diet 13 but did not prevent the development of dystrophy. In this respect, therefore, the fresh, green lettuce differed decidedly from fresh, green alfalfa.

Experiments with diet 13 and vitamin supplements

The curative effect of green alfalfa and the instability of the active factor has made the further study of vitamin supplements desirable. Although diet 13 is deficient in vitamin E, the experiments previously described indicate that avitaminosis E is not the principal factor in the production of dystrophy. The following diets were devised to study this problem further:

a. Diet 204 (diet 13 supplemented with whole wheat germ).
b. Diet 213 (diet 13 supplemented with whole wheat germ treated with ethereal ferric chloride).
c. Diet 214 (diet 13 supplemented with wheat germ oil).
d. Diet 215 (diet 13 supplemented by carotene, or by a combination of carotene and wheat germ oil).
e. Diet 216 (diet 13 supplemented by a combination of wheat germ oil and lettuce, or by a combination of wheat germ oil and dry alfalfa).

a. Diet 204. Whole wheat germ being one of the most potent sources of vitamin E, a diet was made up by mixing 100 gm. of diet 13 with 20 gm. of fresh wheat germ. Rabbits fed this diet were completely protected from dystrophy. However, this does not necessarily mean that vitamin E is the protective agent as some other constituent of the germ may be responsible for the prevention of the dystrophy. Previous experiments have shown that, at least, vitamin E alone is not the preventive factor. The recovery experiments, reported in the next section, must be considered in this connection, since they furnish further evidence of both the preventive and curative action of wheat germ.

In an effort to determine the nature of the effective agents present in wheat germ we performed two sets of experiments. One was concerned with the effect of ethereal-ferric chloride treatment of whole wheat germ, while the other dealt with the effectiveness of wheat germ oil in preventing dystrophy.

b. Diet 213. This diet was like diet 204 except that the whole wheat germ used to supplement the diet 13 had been treated with ethereal-ferric chloride. The results obtained with this diet show that the rabbits were probably maintained for a longer time before becoming dystrophic than on the straight diet 13, although eventually they all became dystrophic. Obviously, the treatment with ethereal-ferric chloride has destroyed or rendered ineffective the protective factor in the wheat germ.

c. Diet 214. Since the vitamin E of wheat germ is found in the wheat germ oil, feeding diet 13 supplemented with this oil should throw light on the role of vitamin E in the prevention of dystrophy as well as help to determine the nature of the protective agent in the whole wheat germ.

The results of these experiments show that the administration of wheat germ oil does not affect the dystrophy producing action of diet 13, except that the onset of the disease is somewhat delayed. Three of the rabbits became definitely dystrophic in 35 to 41 days, one rabbit in 58 days, while another rabbit showed only mild symptoms even after 100 days on this diet. The average time for the onset on diet 214 was 55 days, which is somewhat longer than on diet 13 alone. These experiments with diet 13 supplemented with wheat germ oil corroborate the conclusion that the absence of vitamin E is not the principal factor in the production of muscle dystrophy.

The results with diets 204, 213 and 214 lead, therefore, to the following conclusions: 1) Wheat germ contains a factor which prevents the development of muscle dystrophy in rabbits. 2) This protective factor is destroyed by ethereal-ferric chloride treatment. 3) Cold pressed wheat germ oil does not contain the protective factor necessary to prevent dystrophy.

d. Diet 215. Inasmuch as wheat germ oil was found to be ineffective, while whole wheat germ itself was very effective in the prevention of dystrophy, it seemed reasonable to suppose that a combination of vitamin E with some other factor acts as the protective agent. The work of Aberle ('34) suggests that avitaminosis A may play a part, and we, therefore, arranged a series of experiments with diet 13 supplemented by carotene or by a combination of carotene and wheat germ oil. Since all the rabbits on this modified diet became dystrophic, it is obvious that neither carotene alone nor a combination of carotene with wheat germ oil furnish protection against dystrophy. Experiments in which the diet 13 was supplemented with the vitamin B complex (fresh brewers' yeast) have likewise failed to offer any protection against the development of dystrophy.

The results obtained on diet 13 with various vitamin supplements lead to the conclusion that the dystrophic condition in rabbits on this diet is not due to a deficiency of either vitamins A, B or E, or a combination of vitamins A and E.

e. Diet 216. We have shown previously that wheat germ or fresh, green alfalfa furnish adequate protection against nutritional muscle dystrophy, but dry alfalfa, fresh green lettuce, carotene, wheat germ oil, yeast or a combination of carotene and wheat germ oil are entirely ineffective. In the case of dry alfalfa, lettuce or wheat germ oil the onset of the disease was, however, somewhat delayed as compared with diet 13 unsupplemented by any of these products. These findings suggested that possibly a combination of the various supplements might prove effective and subsequent feeding experiments showed that either a combination of lettuce with wheat germ oil or of dry alfalfa with wheat germ oil is effective in preventing the development of dystrophy in the rabbit. These experiments gain more significance when they are considered in conjunction with the study of curative diets, discussed in the next section, but they definitely point to the conclusion that there are at least two factors concerned in the prevention of dystrophy. Both factors are furnished by

whole wheat germ or by fresh, green alfalfa. On the other hand, one must be found in dry alfalfa or in fresh lettuce while the other is present in the wheat germ oil (cold pressed).

The experiments on the causation and prevention of muscle dystrophy were conducted simultaneously with curative studies. The experiments with the curative diets corroborate the findings reported in this section. A general summary will, therefore, be given at the conclusion of the second section.

II. THE CURE OF NUTRITIONAL MUSCULAR DYSTROPHY

The recovery experiments to be reported here were all carried out according to the same general plan. Muscular dystrophy was developed in rabbits by one of the causative diets, and recovery was attempted by changing the animal to another diet. Whenever this was possible, the state of dystrophy as well as the recovery from dystrophy was checked histologically, biopsies having been performed at suitable stages during the experiment.

Although the results of the microscopic study of the muscles will be presented in a separate paper, it may be pointed out here that, whenever the dystrophic condition was cured by a change in the diet, not only have the outward manifestations characteristic of the behavior of the diseased rabbits disappeared but the muscles themselves have undergone progressive regeneration with ultimate restoration of the normal histological structure.

Experiments with control diet

When the question was first posed whether or not the recovery from dystrophy could be effected, it was decided to place diseased rabbits on an adequate food such as our control diet. Since two dystrophic rabbits made a rapid and complete recovery on this diet, we were convinced that a dystrophic rabbit could be cured of the disease when placed on a proper diet. Naturally, recovery can no longer be expected in animals in which the dystrophy has already progressed

so far that it is unable to eat. The preliminary trials with the control diet led us to secure recovery with the basic diet 13 supplemented with the proper sources of the protective factors.

Diet 13 and green alfalfa

Supplementing diet 13 with 50 gm. of fresh, green alfalfa daily brought about definite recovery from muscular dystrophy, as can be seen from the results recorded in table 1.

TABLE 1

Experiments with diet 13 and green alfalfa as a recovery diet

RABBIT	DIET	DIET SUPPLEMENT	BODY WEIGHT IN GRAMS: At beginning of experiment	BODY WEIGHT IN GRAMS: Maximum weight attained	BODY WEIGHT IN GRAMS: At close of experiment	NUMBER OF DAYS ON DIET: To reach maximum weight	NUMBER OF DAYS ON DIET: During decline in weight	BIOPSY	REMARKS
213	11	—	660	2310	2310	83	0	—	Apparently normal
	13	—	2310	2400	2150	2	5	—	Dystrophic
	13	Lettuce and green alfalfa	2150	2360	2360	30	0	—	Apparently entirely recovered
328	13	—	400	870	840	37	3	I	Dystrophic (++)[1]
	13	Green alfalfa	840	980	980	8	0	II	Definitely improved but still dystrophic (+)
	13	Green alfalfa	980	1890	1890	60	0	—	Apparently entirely recovered

[1] The degree of dystrophy is indicated by the number of plus signs and is based on the estimate of the degenerative process from a microscopic study of the affected muscles.

Diet 13 and lettuce

Encouraged by the recovery effected on diet 13 supplemented with green alfalfa, it seemed advisable to test the effectiveness of other green foodstuffs. We selected for this purpose green lettuce but the results were disappointing and revealed that lettuce alone added to the dystrophic diet 13 does not effect a cure from this condition. These results

substantiated the earlier findings that green lettuce, unlike the green alfalfa, does not contain the necessary factor to prevent the development of muscle dystrophy.

Diet 13 with lettuce and wheat germ oil

Since in the earlier work we found that a combination of lettuce and wheat germ oil was effective in preventing the onset of dystrophy, we studied this combination further by means of curative experiments. The results of these experiments are recorded in table 2. Rabbits 296 and 297 became dystrophic on diet 13 and lettuce, while rabbits 299 and 300 developed dystrophy on diet 13 and wheat germ oil. However, all four animals made rapid recovery when wheat germ oil was added to the former, or lettuce was added to the latter. These results corroborate the previous findings and substantiate further our conclusion that two factors are necessary either for prevention or cure of muscle dystrophy in the rabbit, one factor being furnished by foods like lettuce and the other by wheat germ oil.

Diet 13 and whole wheat germ

To check the results previously described of preventing dystrophy on diet 13 supplemented with whole wheat germ, we performed a series of curative experiments with this substance. The results reported in table 3 show that wheat germ is equally effective both as a preventive and as a curative food. Whole wheat germ, therefore, supplies all the necessary factors for preventing or for curing muscle dystrophy.

Diet 13 with dry alfalfa and wheat germ oil

As a corollary to the earlier experiments, we tried the addition of wheat germ oil to diet 220 (diet 13 supplemented with dry alfalfa) as a curative measure. The results with this diet are summarized in table 4. Rabbit 255 became dystrophic on diet 220, but upon the administration of wheat germ oil it rapidly recovered from the disease. Rabbit 288 developed

TABLE 2

Experiments with diet 13 supplemented with lettuce and wheat germ oil (E) as a curative diet

Rabbit	Diet	Diet supplement	Body weight in grams: At beginning of experiment	Body weight in grams: Maximum weight attained	Body weight in grams: At close of experiment	Number of days on diet: To reach maximum weight	Number of days on diet: During decline in weight	Biopsy	Remarks
280	248	—	560	1500	1230	32	14	—	Pronounced dystrophy
	13	Lettuce	1230	Almost constant weight throughout period		28		—	Still definitely dystrophic
	13	Lettuce and E	1230	2300	2300	91	0	I	Apparently entirely recovered (corroborated histologically)
296	13	Lettuce	500	1690	1600	47	1	—	Dystrophic
	13	Lettuce and E	1600	1700	1620	4	2	—	Apparently improving then sudden paralysis (+)
297	13	Lettuce	500	1040	900	25	6	—	Dystrophy and paralysis
	13	Lettuce and E	900	900	760	0	9	I	Eating and showing improvement. Dystrophic (+++)
	13	Lettuce and E	760	1800	1800	49	0	II	Definite but not complete recovery
	13	Lettuce and E	1800	2120	2120	108	0	III	Practically complete recovery
299	13	E	500	1510	1470	38	3	—	Dystrophic
	13	Lettuce and E	1470	1660	1400	7	3	—	Died—diarrhea. Was making rapid recovery
300	13	E	600	1470	1300	33	5	I	Dystrophic (++)
	13	Lettuce and E	1300	1960	1960	49	0	II	Great improvement but not complete recovery
	13	Lettuce and E	1960	2800	2800	97	0	III	Practically entirely recovered
302	13	A and E	520	1500	1430	38	3	—	Dystrophic
	13	Lettuce and E	1430	1530	1530	6	0	—	Apparently improving then sudden paralysis (++)

TABLE 3

Experiments with diet 13 and whole wheat germ (diet 204) as a curative diet

RABBIT	DIET	DIET SUPPLEMENT	BODY WEIGHT IN GRAMS: At beginning of experiment	BODY WEIGHT IN GRAMS: Maximum weight attained	BODY WEIGHT IN GRAMS: At close of experiment	NUMBER OF DAYS ON DIET: To reach maximum weight	NUMBER OF DAYS ON DIET: During decline in weight	BIOPSY	REMARKS
291	13	—	600	1350	1310	28	3	I	Dystrophic (+)
	13	Wheat germ	1310	2400	2250	55	8	II	Definite recovery (corroborated histologically)
294	13	—	550	1270	1070	26	3	I	Dystrophic (+)
	13	Wheat germ	1070	2060	2060	40	0	II	A good, although not complete recovery
	13	Wheat germ	2060	2900	2900	95	0	III	Complete recovery

TABLE 4

Experiments with diet 13 and dry alfalfa (diet 220) and wheat germ oil (E) as a curative diet

RABBIT	DIET	DIET SUPPLEMENT	BODY WEIGHT IN GRAMS: At beginning of experiment	BODY WEIGHT IN GRAMS: Maximum weight attained	BODY WEIGHT IN GRAMS: At close of experiment	NUMBER OF DAYS ON DIET: To reach maximum weight	NUMBER OF DAYS ON DIET: During decline in weight	BIOPSY	REMARKS
255	220	—	370	1750	1480	47	8	I	Dystrophic (+++)
	220	E	1480	2250	2250	30	0	II	Definitely improving but still dystrophic (+)
	220	E	2250	3180	3180	135	0	—	Apparently entirely recovered
288	13	A and E	650	1860	1680	43	6	I	Dystrophic (++)
	220	E	1680	1980	1450	9	8	—	Apparently slight recovery—then violent onset of dystrophy and sudden paralysis (++++)

dystrophy on diet 13 supplemented with carotene and wheat germ oil. At that stage it was placed on diet 220 with wheat germ oil and showed rapid improvement for 9 days. However, this animal then began to lose weight and within 8 days it suddenly became paralyzed and died. Since this rabbit showed marked recovery for the first 9 days on this diet, the subsequent paralysis and death do not necessarily reflect upon the effectiveness as a curative diet of diet 220 and wheat germ oil.

The results with rabbit 255 again clearly point to the need of two factors for the cure of dystrophy, one of which is supplied by dry alfalfa and the other by wheat germ oil.

SUMMARY AND CONCLUSION

1. Muscle dystrophy in the rabbit could not be prevented by the addition of the following supplements, singly, to diet 13: Dry alfalfa, a vegetable oil, lettuce, vitamin A (carotene in oil), vitamin E (cold pressed wheat germ oil), or vitamin B (yeast).

2. Neither the omission of the ethereal-ferric chloride treatment nor the substitution of aqueous-ferric chloride treatment of the basic diet entirely abolished its dystrophy producing effects.

3. Prevention of muscle dystrophy as well as cure of the dystrophy already developed was effected by feeding the following supplements along with the dystrophic diet 13: Fresh green alfalfa, lettuce and vitamin E (wheat germ oil), dry alfalfa and vitamin E (wheat germ oil) or whole wheat germ.

The experimental results lead to the conclusion that there must be at least two factors involved in the prevention or in the cure of muscle dystrophy. Both factors are present in fresh green alfalfa or in whole wheat germ. On the other hand, one of these factors is supplied by wheat germ oil (cold pressed), while the other is present in lettuce or in dry alfalfa. At least one of the factors is easily destroyed by ethereal-ferric chloride, by drying or by extraction with water or alcohol.

NOTE ON THE OCCURRENCE OF PARALYSIS

Occasionally in the course of an experiment an amyotonic condition suddenly developed in our animals which resembled flaccid paralysis. This condition was characterized by sudden onset, very rapid progress and usually fatal termination.

This peculiar paralytic condition developed in some of our rabbits on diet 13 alone, on diet 13 with wheat germ oil, diet 13 with carotene and wheat germ oil, diet 13 with lettuce and wheat germ oil, and on diet 13 with dry alfalfa and wheat germ oil.

In rabbits 285, 286, 290 and 304, which were on a dystrophy producing diet, the paralysis apparently developed independently of the dystrophy. Microscopic examination of the muscles showed advanced lesions in rabbits 285 and 286, while in rabbits 290 and 304 only the earliest signs of dystrophy were present, but the paralysis in all four animals was marked and severe. Thus, the severity of the muscle dystrophy and that of the paralysis did not run parallel. The paralysis, furthermore, resembled in no way the dystrophic condition outwardly, and the affected animals assumed a sprawling position with their limbs flaccid and extending sidewise.

In some animals (rabbits 296 and 302) the paralysis appeared while they were recovering from a previous attack of dystrophy. These animals had developed definite dystrophy and were shifted to a curative diet, on which they already were making marked improvement. The microscopic examination of their muscles actually revealed regenerative recovery when they were suddenly stricken by the paralysis.

Rabbit 297 on diet 13 supplemented with lettuce became dystrophic and paralyzed at the same time. This animal was still able to feed and recovered ultimately when wheat germ oil was added to the above diet. Three biopsies were performed on this animal during its recovery, which fully corroborated the findings from observation of the animal's behavior.

Ringsted ('35) described paresis appearing in rats suffering from chronic avitaminosis E which is quite similar to the neuropathic disturbance noted in vitamin A deficient rats by Aberle ('34). The paresis studied by Ringsted developed in adults, was of the relaxed type and was accompanied by disturbances of deep-seated sensibility much more than of the cutaneous sensibility of the legs and tail. Aberle did not observe changes in sensibility or atrophy of the skin and fur, as was the case in Ringsted's animals.

The variety of diets upon which paralysis developed in our rabbits makes it very difficult to assign its causation to any particular factor. The one thing common in all our cases is the use of diet 13; however, only a small proportion of the rabbits on this diet or variations of this diet have actually become paralyzed.

Our findings seem to point out that whatever may have been responsible for paralysis bears no direct relation to the causation of muscle dystrophy. The paralytic condition occurred sporadically in animals either affected by dystrophy or while they were recovering from the dystrophy, and is in all probability an entirely unrelated disorder.

The fact that paralysis occurred only among the rabbits from three litters, which were obtained from the same rabbitry, suggests the possibility that we are dealing with an hereditary disability. The extreme suddenness of the onset of the paralysis and the rapidity with which it usually terminated in death made it impossible to study the paralyzed animals systematically.

Summary of Feeding experiments

Causative diets — Onset of dystrophy: *Rapid*	*Causative diets* — Onset of dystrophy: *Slow*	*Preventive diets*	*Curative diets*
Diet 13	Diet 11 (untreated diet 13)	Rabbit chow	Rabbit chow
Diet 13 treated with aqueous-ferric chloride	Diet 13 + wheat germ treated with ethereal-ferric chloride	Diet 13 + wheat germ	Diet 13 + wheat germ
Diet 13 with mazola instead of lard	Diet 13 + E	Diet 13 + lettuce + E	Diet 13 + green alfalfa
Diet 13 + alfalfa extracts	Diet 13 + E + A	Diet 13 + lettuce + E + A	Diet 13 + lettuce + E
Diet 13 + yeast	Diet 13 + lettuce	Diet 13 + dry alfalfa + E	Diet 13 + dry alfalfa + E
Diet 13 + A	Diet 13 + dry alfalfa	Rabbit chow treated with ethereal-ferric chloride (diet 201)	
		Diet 201 + E	
		Diet 201 + E + A	

E = cold pressed wheat germ oil, A = carotene in oil.

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CHANGES IN THE VAGINAL EPITHELIUM OF THE RAT ON AN EXCESSIVE VITAMIN A DIET [1]

T. C. SHERWOOD, M. A. BREND AND E. A. ROPER
Department of Anatomy and Physiology, University of Kentucky, Lexington

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Many investigators have reported that excessive cornification, in the vaginal epithelium of the albino rat, is an early manifestation of vitamin A deficiency, Evans and Bishop ('22), Baumann and Steenbock ('32), Aberle ('33), Mason and Ellison ('35).

Evans ('28) noticed that four-fifths of the copulations were unsuccessful due to a lack of proper implantation. He also reported that only about one-fifth of the copulations lead to the birth of litters.

Sherwood, Toth and Carr ('34), Sherwood and Luckner ('35) reported a change in the colloidal content of the thyroid gland and a change in the histological picture when large amounts of cod liver oil, haliver oil and, carotene were administered. It was therefore concluded that the excessive vitamin A added to the normal diet was responsible for a greater activity of the epithelial tissues.

EXPERIMENTAL PROCEDURE

Since the vaginal epithelium was altered as reported by Mason and Ellison ('35) when a diet deficient in vitamin A was administered, it was deemed of sufficient interest to investigate the effect of large amounts of vitamin A on the vaginal tissues.

[1] The carotene used in the present investigation was generously furnished by S. M. A. Corporation, Cleveland, Ohio.

The technic of Long and Evans ('22) was used in the study of the vaginal smear picture. The source of vitamin A was carotene. Corn oil as well as cottonseed oil was used for the control animals. Since cottonseed oil is used as a base for carotene, it was thought necessary to investigate the effect of this oil in order that the results could be interpreted as being due to the carotene or provitamin, and not to the oil.

A group of eighteen animals, 150 days of age, were examined at 8-hour intervals for the normal vaginal smear content until eight complete oestrous cycles had been determined. These animals were then placed on 1500 international units of carotene daily, in addition to the normal diet, for a period of 15 days. The vaginal smear technic was continued during the carotene administration. Vaginal smears were examined after the carotene was discontinued until eight normal oestrous cycles had again been observed.

A second group of sixteen albino rats, 150 days of age, were used and the procedure repeated as mentioned above with the exception that 3750 international units of carotene were given daily for a period of 15 days.

Both experimental and control animals were fed dried skim milk, dried meat, cod liver oil, wheat germ, yellow corn grits, black-strap molasses, iodized salt, organic minerals, wheat cereal, oat cereal, corn cereal and vegetable fiber. This material was given in the form of a stock dog food[2]. This diet was supplemented with fresh whole milk and green lettuce.

RESULTS

The control animals showed no change in the vaginal smear even though they had received 0.5 cc. of cottonseed oil daily for a period of 15 days. The rats that were administered corn oil did not show a change in the vaginal picture. These rats demonstrated an oestrous cycle of approximately 4½ days in every case.

[2] Purina dog chow obtained from the Purina Co., St. Louis.

Table 1 represents the averages of both control and experimental animals. Five individual animals of each experimental group are also presented for the purpose of showing animal variations. The vaginal smears were observed to be abnormal within 2 days following carotene administration. The smears did not progress from the nucleated epithelial to the cornified cell stage.

TABLE 1

The effect of vitamin A given in the form of carotene on the vaginal smear picture

RAT NO.	NORMAL CYCLES	AVERAGE DAYS	DAYS ABNORMAL	NORMAL CYCLES	AVERAGE DAYS	REMARKS
1	8	4.5	32	7	4.5	Animals 1–5 1500 international units daily for 15 days
2	4	4.4	33	5	4.5	
3	6	4.5	34	4	4.6	
4	6	4.5	34	5	4.7	
5	7	4.4	34	5	4.6	
6	8	4.5	34	8	4.4	Animals 6–10 3750 international units daily for 15 days
7	8	4.5	31	5	4.0	
8	5	4.5	31	5	4.0	
9	5	4.5	31	5	4.4	
10	7	4.5	31	5	4.2	
11	5	4.2	—	5	4.2	Cottonseed oil controls
12	5	4.2	—	5	4.2	
Average of animals on 1500 units of vitamin A—18 rats						
	7	4.3	34	6	4.5	
Average of animals on 3750 units of vitamin A—16 rats						
	6	4.4	31	5	4.2	

Upon examination of the vaginal smears an excess of nucleated epithelial cells were seen, regardless of the phase of the oestrous cycle. Leucocytes were also observed continuously but were not as numerous as were the epithelial cells. Occasionally a few scattered cornified cells were observed which might have been obtained from the orifice of the vagina.

The normal oestrous cycle was not observed for approximately 20 days after the carotene feeding had been discontinued.

DISCUSSION

Baumann and Steenbock ('32) observed dioestrous smears within 1 week and oestrum in 2 weeks after the administration of carotene to rats that had been held on a diet deficient in vitamin A. The rapid return to normal seems to indicate a powerful vitamin effect on the growth of epithelial tissues.

The results obtained in the present investigation seem to be due to a rapid cell growth as indicated by the large number of young nucleated epithelial cells which predominated throughout the experimental phase of the problem.

The carotene must have been converted into vitamin A in excessive amounts since the rapid growth was noticed within 2 days after experimentation had begun.

Since the control rats gave no indication of a changed vaginal smear picture, it is clearly demonstrated that the change must have been brought about through the effect of large amounts of vitamin A in the experimental animals. This phenomenon must be expected since vitamin A has an effect on the growth processes of the animal in general. However, it is not to be expected that any normal growing process would cause a change in the oestrous cycle as abnormal as reported in the present investigation.

Further studies are being made for the purpose of finding a possible effect elsewhere in the organism which might be directly or indirectly responsible for the changes observed in the vaginal smears. The animals did not show a desire to copulate in most instances. When the animals did copulate, no pregnancy resulted until the vitamin effect had disappeared. One rat was left with the male for a period of 10 days and still gave the abnormal vaginal smear picture.

SUMMARY

Carotene in oil given daily for 15 days and representing as many as 3750 international units of vitamin A prevented directly or indirectly a normal vaginal smear picture.

Control rats that had received only the normal diet showed a normal oestrous cycle throughout the investigation.

Control rats given cottonseed oil and corn oil also exhibited a normal vaginal picture as observed by the smear technic.

Carotene in large amounts produced a rapid growth of the epithelium for a period of approximately 30 days. This was indicated by the large number of young nucleated epithelial cells which predominated in the vaginal smears.

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THE VITAMIN C CONTENT OF HUMAN MILK AND ITS VARIATION WITH DIET [1]

IVA SELLEG AND C. G. KING
Department of Chemistry, University of Pittsburgh, Pittsburgh

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The primary purpose of the present investigation was to find the approximate antiscorbutic value of human milk. There were important and closely related items which could be studied simultaneously, however, such as the range of variation in individual cases and the influence of wide variations in vitamin intake during short experimental periods. Of particular interest was the possibility thus afforded of establishing directly the quantity of vitamin C which could be considered normal for an infant.

EXPERIMENTAL

Method of analysis. The 2,6-dichlorophenol-indophenol titration technic was used, essentially as described by Bessey and King ('33). The method has been shown to give an accurate measure of the ascorbic acid content of most normal tissues of both plants and animals, and in an earlier investigation in our laboratory (Waugh and King, '33) it was found that the indophenol titration of cows' milk was accurate within the limits of biological assay. We found it unnecessary to precipitate the curd and filter before titrating, because the direct addition of 8 per cent trichloroacetic or acetic acid followed by dye titration gave satisfactory end points with a shorter time interval and less manipulation. Duplicate or triplicate analyses were made on 4 or 5 cc. portions of each

[1] Contribution no. 311 from the department of chemistry, University of Pittsburgh.

sample. Samples were obtained from one to three times per day and placed in refrigeration in aluminum capped test tubes whenever held during brief periods before titrating. The orange juice given as a vitamin supplement[2] was titrated regularly to provide an accurate record of the total vitamin intake provided in the special supplement.

Clinical records. The patients from whom samples were obtained had been under medical care long enough to give reasonable assurance of normal health. Their diets previous to hospitalization had apparently been representative for the community, and the hospital dietary was probably above the average in general quality. Most of the patients included in

TABLE 1

Vitamin C content of human milk (milligrams per cubic centimeter) and the effect of a special vitamin supplement

AVERAGE DAILY VITAMIN SUPPLEMENT	NUMBER OF CASES	AVERAGE AGE, YEARS	INITIAL VALUES 3 TO 6 DAYS	DAYS POST PARTUM					
				5	6	3	8	9	10
None	17	24.3	0.053	0.056	0.058	0.060	0.066	0.064	0.064
210 mg.	19	24.7	0.056	0.067	0.070	0.068	0.071	0.072	0.073
430 mg.	17	25.5	0.055	0.065	0.070	0.076	0.077	0.078	0.081

the study were given special supervision[3] to avoid dietary irregularities and to insure adequate care in recording the administration of orange juice and in obtaining samples. The cooperation of the patients was purely voluntary.

Experimental data and discussion. A summation of the major part of the investigation is given in table 1. It will be noted that the titration values for the three groups were comparable at the beginning of the test, and that there was a moderate but distinct rise in the antiscorbutic value of the milk from patients who received no special vitamin C supplement. This might be interpreted as a normal physiological

[2] We are indebted to the California Fruit Growers' Exchange for supplying the oranges as needed.

[3] The study was made possible by the generous cooperation of Dr. B. Harden, Miss Trumbull, superintendent, and other members of the staff of the Elizabeth Steele Magee Hospital, to whom the authors are greatly indebted.

change in the composition of the milk secreted, but a more careful evaluation of the data indicates that it represented the rise made possible in large part by the improved hospital dietary compared to the dietary before hospitalization. The number of instances where the titration values remained practically constant throughout the period of observation gave evidence that there would not have been such a distinct rise independent of increased dietary intake. The vitamin content of colostrum, based upon twenty cases, showed wide variation, but gave an average value that was comparable with normal milk. The slowness of obtaining samples during the first few days of lactation no doubt occasioned greater losses by atmospheric oxidation. For example, the average loss in titration value for eleven samples held in a refrigerator during 18 hours was 27.2 per cent. This was much greater than the loss observed previously for bottled market milk (Waugh and King, '33).

The relatively slow and limited rise in antiscorbutic value when patients received a special supplement of orange juice equivalent to 210 or 430 mg. of ascorbic acid per day is noteworthy in that it provides an indication of a maximum and approximately optimum level of secretion, above which an excessive dietary intake results chiefly in a rapid urinary excretion without disturbing the lactation level. It was not possible, with the limited facilities and personnel available to run regular and immediate titrations of urinary output. However, the titration data for four patients in the control group, five receiving a supplement of 165 mg. of vitamin per day, and ten receiving a supplement of 475 mg. of vitamin per day, showed eliminations of not less than 18, 48 and 296 mg. of vitamin per day respectively. These values are probably somewhat low due to unavoidable delays in titration. The oxidation loss is very rapid in alkaline urine. Similar rapid urinary excretion has been shown by Harris and Ray ('35) and a number of other investigators. Harris and Ray reported daily excretions of 11.7 to 38 mg. Marked differences in 'kidney thresholds' in relation to ascorbic acid excretion

probably account in large degree for the individual differences observed occasionally in the rate of depletion and protective requirement (both human and guinea pig).

There was a noticeable variation in the antiscorbutic value of the milk at different times of the day and from day to day, but the average difference between morning and afternoon samples was small. For 104 samples collected between 9 and 11 A.M., compared with the same number collected from the same patients on the same days between 3 and 6 P.M., the average values were 0.060 and 0.064 mg. per cubic centimeter respectively. The individual values for four patients living at home (average age of children, 43 days) and receiving representative dietaries were 0.062, 0.061, 0.055 and 0.053 mg. per cubic centimeter. The extremes of individual variation observed at any time were 0.0124 and 0.1081 mg. per cubic centimeter. The average value reported from thirteen cases by Harris and Ray ('35) during the course of this investigation was 0.056 mg. per cubic centimeter (lactation time was about 3 months).

TABLE 2

Increased vitamin content of milk, subsequent to receiving a special supplement of 500 cc. of orange juice (310 mg. of vitamin C) per day

PATIENT	INITIAL VALUE	SUCCESSIVE DAYS		
		1	2	3
	mg. per cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*
A	0.026	0.032	0.054	0.069
B	0.043	0.053	0.074	0.078
C	0.032	0.041	0.053	0.065
D	0.044	0.048	0.070	0.088
E	0.043	0.052	0.062	0.074
F	0.019	0.019	0.021	0.032

In every case where the initial value was subnormal, and vitamin supplements were given over a period of 3 days or more, a distinct rise became evident. Table 2 illustrates the typical rapid response to a generous vitamin intake when initial values were distinctly subnormal. This appears to provide reasonably good evidence that the previous dietaries

had been inadequate for normal or optimum lactation in regard to this specific factor. The last patient listed in table 2 stated that she had eaten practically no citrus fruits or other noteworthy antiscorbutic foods, and that her major foodstuffs (before being admitted to the hospital) were rice and macaroni.

The normal level of vitamin C in human milk is strikingly higher than in cow's milk. Different investigators have reported varying results concerning the antiscorbutic value of cow's milk (associates of L. A. Rogers, '35; Riddell, Whitnah, Hughes and Lienhardt, '36; Sherman and Smith, '31), but a summary of the evidence would indicate that 25 to 30 cc. may be considered a minimum protective level for guinea pigs under optimum conditions of vitamin intake (spring pasturage). This would indicate an ascorbic acid content of approximately 0.02 to 0.025 mg. per cubic centimeter which is in good agreement with the titration data cited by Hughes and associates and Harris and Ray. Under general feeding and merchandizing conditions the average value for raw market milk is more nearly 0.010 mg. per cubic centimeter and secondary oxidation may decrease this readily to extremely low values. Since cattle are apparently capable of synthesizing all or the greater part of their vitamin C requirement (Thurston et al., '29), it is not surprising to find a lower lactation level. The general need for providing a special vitamin C supplement (i.e., in addition to cow's milk) for infant feeding is made evident by the above data.

It seems evident that the normal requirement of vitamin C per day for an infant may be estimated from the amount supplied in mothers' milk when the parent is receiving a good diet. On this basis, with a fluid intake of 21 ounces, the normal vitamin C supply would be approximately 40 mg. per day. In many cases, on a representative hospital diet without a special supplement, the intake would be 50 mg. per day. With increasing food consumption by the infant the normal intake would be correspondingly higher.

In the light of the above data concerning the low vitamin level of human milk when the mother's diet has not been adequate, and earlier data (Yavorsky, Almaden and King, '34) showing that occasionally infants are very deficient in vitamin C tissue reserves, the suggestion that infants and embryonic tissue generally may not need a dietary source of the vitamin (Rohmer et al., '35; Banerjee, '35) is very unfortunate. There is no clear-cut evidence to support the view that either guinea pig or human tissues can synthesize vitamin C from non-vitamin food material at any stage in their development.

TABLE 3

Relative constancy of vitamin C in milk when the initial lactation level was high, and when a special supplement was given

VITAMIN SUPPLEMENT	AVERAGE INITIAL LEVEL (3 TO 6 DAYS)	VITAMIN CONTENT ON SUCCESSIVE DAYS		
		1	2	3
mg. per day	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*
165	0.081	0.083	0.083	0.081
250	0.075	0.075	0.071	0.073
375	0.080	0.081	0.081	0.082
375	0.083	0.082	0.085	0.087
500	0.072	0.083	0.087	0.088
500	0.072	0.080	0.083	0.083
500	0.073	0.071	0.078	0.077

The role of vitamin C in maintaining both resistance to infections (Clausen, '34) and protection against the injury of bacterial toxins (King and Menten, '35; Rinehart and Mettier, '34; Schultz et al., '35) indicates the importance of avoiding depletion of the tissue reserves. Without a special antiscorbutic supplement, the vitamin C reserves of formula-fed infants would be depleted steadily and an impairment of health would follow in corresponding degree.

SUMMARY

The vitamin C content of human milk was found to vary from 0.012 to 0.108 mg. per cubic centimeter, the average of fifty-three cases, 3 to 6 days post partum, being 0.055 mg. per cubic centimeter. On a good hospital dietary without

special supplements the average value rose gradually to 0.064 on the tenth day. The values for two groups receiving orange juice supplements equivalent to 210 and 430 mg. of vitamin C per day rose to 0.073 and 0.081 mg. per cubic centimeter respectively on the tenth day. When the mother was receiving an adequate diet the usual range of vitamin C was in the zone of 0.060 to 0.080 mg. per cubic centimeter. This indicates that the normal vitamin C intake for an infant is approximately 40 to 50 mg. per day during the first few weeks of life. Several cases with markedly subnormal antiscorbutic values were observed, and these rapidly approached normal when an orange juice supplement was given. Patients with initial high lactation levels indicative of a good state of nutrition showed relatively small increases when vitamin supplements were given. The excess quantities of vitamin were eliminated rapidly in the urine. The present investigation and data reported previously provide strong evidence against the suggestion that guinea pigs and humans can synthesize adequate quantities of ascorbic acid during gestation or infancy.

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LAFAYETTE BENEDICT MENDEL—AN APPRECIATION [1]

WILLIAM C. ROSE
Laboratory of Biochemistry, University of Illinois, Urbana

In undertaking to speak of Professor Mendel and his work, I do so with a profound sense of humility, fully realizing that nothing I may say can add to the fame he has achieved, or deepen the esteem in which he is held by each of you. But despite this fact, I welcome the opportunity of paying my respects to one whom I have had the privilege of knowing intimately as teacher and friend.

Early in his career Lafayette Benedict Mendel demonstrated his unusual talents. In 1893, at the age of 21 years, he received the doctorate from Yale University. The following spring his thesis, published with Prof. Russell H. Chittenden, appeared in the Journal of Physiology. It is entitled, "On the Proteolysis of Crystallized Globulin." It deals with the separation and characterization of the proteoses and peptones produced by the action of pepsin-hydrochloric acid upon edestin. The paper is among the first to describe the enzymatic hydrolysis of a *crystalline* protein.

Since the publication of this thesis, more than 300 contributions have been made by Professor Mendel and his associates. Obviously, it will not be possible for me to review them in the time available. Furthermore, to do so would be quite

[1] This brief paper was prepared at the request of the Council of the American Institute of Nutrition, and was read at the annual meeting of the Institute in Washington, D. C., March 25, 1936.

It was not the purpose of the writer to give an account of the life and work of Professor Mendel. The intention was rather to emphasize the remarkable personal attributes of one who was a great leader and an inspiring teacher. The paper is published at the suggestion of the editorial board of the journal.

presumptious inasmuch as you are already familiar with their contents. However, it may be of interest, particularly to the younger members of this audience, to list some of the topics which he investigated, especially those which repeatedly attracted his attention. Of these, the following may be mentioned: The chemical changes during embryonic development; the alimentary processes, including the secretory activity of glands, the action of enzymes, and the paths of absorption of the digestive products; the formation and flow of lymph; the permeability of cell membranes; the regulation of blood volume; the metabolism of carbohydrates, proteins, fats, purines, pyrimidines, creatine, creatinine, and sulfur; the paths of excretion of inorganic salts; the rate of elimination of nitrogen under widely diversified conditions; the nutritive value of cereals and other natural food materials; and the utilization of purified carbohydrates and proteins. In collaboration with the late Dr. Thomas Burr Osborne, Professor Mendel published more than 100 papers upon growth, with particular reference to the importance of food 'accessory factors' and amino acids. His extensive investigations upon vitamins A and B, and the role of lysine, tryptophane, and cystine in growth are classics in the literature of nutrition.

An examination of Professor Mendel's contributions cannot fail to impress one with the intellectual resourcefulness of the man, and his remarkable versatility in research. Scarcely any aspect of nutrition escaped his scientific curiosity. Unusually gifted as a writer, he was as judicious in the preparation of his published reports as he was meticulous in the conduct of his laboratory investigations. The logical development of subject matter, clarity of expression, and forceful diction render his papers models of scientific literary perfection.

But it is not my purpose at this time to discuss Mendel the investigator and literary genius, but rather Mendel the teacher, the counselor, and the friend of students. These attributes possibly are less clearly appreciated by those who

have not worked under his direction. From year to year Professor Mendel attracted to his laboratory an unusually large number of young men and women. The departmental records, made available through the courtesy of Prof. Arthur H. Smith, show that ninety-two students received the degree of doctor of philosophy under his direction. In addition, 237 graduate students and ninety-six advanced research fellows and visitors received part of their training under his supervision.

Professor Mendel created a truly remarkable intellectual atmosphere in his laboratory. Though always kind and sympathetic in his relations with students, yet he had a ready faculty of impressing newcomers promptly with the conviction that their sole reason for being there was to accomplish something. An indefatigable worker himself, he rightly expected others to make full use of their opportunities. He had little time for the laggard, but exercised infinite patience and tact in dealing with those who were industrious and reasonably intelligent. During that period of his career with which I am most familiar—the period which the very recent graduates sometimes denote, to my chagrin, as the 'early days'—the professor practically lived with his students. He liked to watch them work, to observe how they went about their tasks; and each day, unless prevented by some administrative or other duty, he 'made his rounds,' as he said. His presence was always inspiring. During these frequent and informal conferences the students really came to know him, and to appreciate his unselfish spirit and whole hearted interest. If carelessness, lack of neatness in work, or poor technic was observed, it was immediately corrected; if encouragement was required, it was cheerfully given. With the beginner in research he emphasized the disciplinary value of unsuccessful experiments. Apparent failures never were to be taken too seriously, but rather were to be made use of in devising more satisfactory methods of attack. The relationship which existed between 'The Professor' and those working under his guidance is exemplified by the manner in which he invariably

referred to the group as his 'laboratory family.' As the years passed, and his keen intellect and mature judgment were more widely recognized and sought, his university, as well as innumerable outside committees and organizations, made greater and greater demands upon his time and energy. But despite the enormous responsibilities which rested upon him the welfare of his students was always uppermost in his mind.

Professor Mendel possessed an intimate and detailed comprehension of nutrition which was little short of astounding. He was always a prodigious reader. For this purpose, many evening hours, and at least one full afternoon per week were reserved. Any of his older students will recall how on Thursday afternoons he sat in the reading room of the Old Library completely absorbed in his work, and totally oblivious to everything about him. Early in his career he had established a regular habit of broadening his personal knowledge of the work of others. This became a lifelong practice. Once he remarked casually that while he was a student in Baumann's laboratory at Freiburg he utilized his 'spare time' by reading every paper in the first twenty-two volumes of Hoppe-Seyler's Zeitschrift. But even more remarkable than his capacity for reading, was his uncanny ability to remember what he had read. A personal experience will serve to illustrate this point. One day, in the course of his regular interviews with the graduate students, he came to my desk. After the usual inquiries as to the progress being made, I mentioned a difficulty encountered in the conduct of the investigation. The particular problem to which I referred has long since escaped my memory, but 'The Professor's' answer will never be forgotten. Without a moment's hesitation he said, "Oh, you will find a paper on that very subject in volume 17 of the Biochemische Zeitschrift." And then as he walked away he turned and added, "That paper begins about page 563." Needless to say, I was left gasping with astonishment. But the incident was not a unique one. Other students of his have had like experiences.

Much could be said regarding Professor Mendel's methods of class instruction. During the period to which I refer, the enrollment in his general course in physiological chemistry was relatively small, inasmuch as the medical students were taught as a separate group. The class periods were not divided into lecture and laboratory hours, and were used as conditions demanded. Lectures varied in length from a few minutes to 2 or more hours depending upon the importance and complexity of the subject under discussion. Quizzes, both oral and written, were given at frequent intervals, and usually without previous notice. Generally, part of the laboratory assignments were made at the beginning of the class period. 'The Professor' dictated the directions. Thus the romance of experimentation was not destroyed by the use of a laboratory manual containing the results to be expected in each manipulation. After a suitable interval, the students were called together for a conference in which their observations were checked and criticized. The climax of each period was 'The Professor's' summary of the day's accomplishments. In the pertinent and telling fashion of one thoroughly versed in the art of precise thinking, he emphasized the salient points and their applications. In the words of Macauley, "He had a wonderful talent for packing thought close, and rendering it portable." No student, save a moron, could experience one of those class periods without acquiring new enthusiasm for the subject in which he was being trained.

The high point of each week's activity was the seminar. It was then that the 'laboratory family' gathered to partake of a 2-hour intellectual feast planned and supervised by 'The Professor.' Only those of the immediate 'family' were permitted to sit at the table. An outer row of chairs provided places for the transients—that is to say, the visitors from other departments, and the out-of-town guests. Each member of the household was required to contribute to the 'meal.' From the more mature young men and women to the younger 'children' of 'the family,' the contributions were carefully adapted to the 'digestive' capacities of the individuals. Correct table technic was expected, and few reprimands were

necessary; but woe unto him who spoiled the 'meal' by inadequate preparation! The kindly figure at the head of the table *could* become exceedingly stern. The mimeographed programs constituted the menus. Occasionally they appeared in English or French, but more frequently in German. Sometimes they provided historical 'food for thought,' as represented by the contributions of a single distinguished investigator, or the development of an important theory. Perhaps more generally they were formulated from the 'articles of diet' in the current literature. Whatever their composition they were always well balanced, and capable of ready assimilation, if properly prepared.

On these weekly occasions Professor Mendel was at his best. He literally radiated enthusiasm. By skillfully directing the discussion of the students, and by his own well chosen remarks, he placed each contribution in its proper setting with respect to the subject as a whole. His wide personal acquaintance with the leading investigators abroad enabled him frequently to relate some interesting or amusing incident about the authors whose papers were being discussed. He never failed to see, or make the most of, the humor in any situation. I recall vividly an occasion of this sort. A paper by a distinguished German physiologist had just been presented by a member of the class. The author's name brought to Professor Mendel's mind an incident which occurred while he was a student in Germany. It seems that the professor of physiology was demonstrating to his class the effect of stimulating a certain nerve. The nerve was small and rather difficult to locate. Although he had performed the experiment many times before, he was experiencing considerable embarrassment on this particular day, for the nerve actually seemed to be missing. The situation was becoming tense. Suddenly his Diener had an inspiration, and in a tone loud enough for the students to hear remarked, "Herr Geheimrat, da is der Nerv!" "Unsinn," said the Geheimrat, "Was wissen Sie von dem Nerv?" "Ja, ja," said the Diener, "Sie haben recht, Herr Geheimrat; aber da is der Nerv." Thus the great

physiologist was humiliated by his Diener. But this story is not the one I wish to emphasize. The sequel is the important matter. After relating the incident, Professor Mendel, with a merry twinkle in his eyes and a faint suggestion of a smile remarked, "You know, one German Diener is more help in a laboratory than are two American assistants." An indelible impression was made on my mind; and well might it, for at that very time I happened to have been 'The Professor's' laboratory assistant.

To his students Professor Mendel was more than a distinguished scientist and a great teacher. Somehow, he directed the aspirations and broadened the perspective of those who came under the charm of his personality. He was not content merely to impart facts to, or to perfect the scientific technic of those about him. These things he did; but in addition, he implanted ideals—ideals of tolerance, unselfishness, intellectual honesty and service. Helmholtz, in speaking of his noted teacher, Johannes Müller, said, "Whoever comes into contact with men of the first rank has an altered scale of values in life." No more appropriate tribute than this could be paid to our distinguished friend whom we honor on this occasion. His students saw him as the personification of the ideals which they admired. They caught his spirit; and determined, perhaps unconsciously, to 'carry on' in his way. Because of these attributes so difficult to describe, and yet none the less real, Mendel won and retained the confidence, the respect, and the devotion of his pupils. He became their guide and counselor. Recently, one of his former students wrote, "It is our task to continue his ways of doing things, for we have learned their value." Another said, "It will be difficult for some of us to plunge ahead without his encouragement." And still another remarked, "I think those of us who knew him longest will miss him most." As we contemplate Mendel's achievements, and evaluate anew his great contributions to the science of nutrition, we may anticipate with confidence that his work will continue in the lives of the multitude of young men and women whom he trained and inspired.

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SUPPLEMENT

PROCEEDINGS OF THE THIRD ANNUAL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

MINUTES OF MEETING OF AMERICAN INSTITUTE OF NUTRITION, WASHINGTON, D. C., MARCH 25, 1936

The third annual meeting of the American Institute of Nutrition was held in Washington, D. C. at the Hotel Washington on March 25, 1936 as per copy of program. Although only 97 members and 178 guests registered there were between 400 and 500 in attendance during the entire program.

President John R. Murlin presided at the scientific sessions and business meeting. The meeting started promptly at 9.30 A.M. and each speaker was gracious enough to confine his remarks to the time allotted for the presentation of his paper. This helpful cooperation on the part of all the speakers enabled the entire program to move according to schedule and therefore with ease, satisfaction and enjoyment. All the papers listed on the program were given.

The business meeting at 11.30 A.M. was called to order by President Murlin. The reading of the minutes of the preceding meeting was dispensed with since they had been published in The Journal of Nutrition and could be read by all. The president announced that any comments on the minutes as published could be sent in writing to the secretary.

The report of the treasurer was read by George R. Cowgill. It was moved by David Drabkin and seconded by J. H. Jones that the report of the treasurer be accepted. The motion was carried. David Drabkin and Martha Koehne were appointed auditors. After examination of the treasurer's report they declared it correct.

President Murlin reported the following recommendations as made by the council:

That the annual assessment be $1.00 per member.

That the American Institute of Nutrition should continue for the present to remain an independent unit and not apply for membership in the Federation. The council had considered the various courses open in the future development and possible enlargement of the society which might render a greater service to those interested in nutrition. The annual meeting for 1937 was set as customary for the day preceding the meeting of the Federation of Societies for Experimental Biology and Medicine to be held in Memphis, Tenn.

That the present method of selecting papers for the scientific program be continued. It was explained that each of the seven council members selected independently the papers that in his judgment represent the most important scientific contributions to the advancement of the science of nutrition. Due consideration is also given to: Type of subject matter, a concise abstract of the relevant facts, a well-balanced distribution of papers covering human and animal nutrition, the number of times the applicant has appeared previously on the program, the ability of the speaker to present subject matter in an intelligible and interesting manner; and members are given preference to non-members. The high agreement of the members of the council in their independent choice of papers has demonstrated that the final program represents an unbiased opinion.

That Prof. Russell H. Chittenden be made an emeritus member of the Institute. It was so moved by Henry C. Sherman and seconded by Eugene F. DuBois. The motion carried.

That the fifteen following candidates be elected to membership in the American Institute of Nutrition:

C. P. Berg	J. E. Hunter	F. Santos
M. Bruger	O. L. Kline	F. W. Schlutz
J. S. Butts	A. Knudson	T. S. Sutton
D. B. Dill	J. P. Outhouse	H. C. Trimble
T. G. H. Drake	W. Peterson	H. H. Williams

It was so moved by R. Adams Dutcher and seconded by F. F. Tisdall. The motion was carried unanimously.

The following members, having reached 65 years of age during the society's year, 1935–1936, became emeritus members by operation of the society's rule:

F. G. Benedict	E. P. Joslin	A. E. Taylor

R. Adams Dutcher, chairman of the committee on nomination of officers, reported names for the various offices provided in the constitution as follows: President, Eugene F. DuBois; vice-president, Wm. Boothby and Mary Swartz Rose; secretary, Icie G. Macy; treasurer, George R. Cowgill and H. H. Mitchell; councillor, G. E. Cullen and C. A. Elvehjem; members of the editorial board, W. H. Griffith, A. C. Ivy,

F. C. Koch, Grace MacLeod, E. M. Nelson and L. H. Newburgh. The report of the nominating committee was accepted. On the motion of Henry C. Sherman and seconded by T. M. Carpenter the secretary was instructed to cast a unanimous ballot for Eugene F. DuBois for president. In like manner on the motion of L. A. Maynard and seconded by Henry C. Sherman the chairman was instructed to cast a unanimous ballot for Icie G. Macy for secretary. The president and secretary were declared duly elected. The remaining officers were elected, namely: Vice-president, Mary Swartz Rose; treasurer, George R. Cowgill; councillor, C. A. Elvehjem; editorial board, E. M. Nelson, F. C. Koch, Grace MacLeod and L. H. Newburgh. F. C. Koch was appointed to fill the unexpired term made vacant through the death of Prof. Lafayette B. Mendel. A. D. Holmes and M. Elizabeth Marsh served as tellers.

In accordance with the constitution the chairman named the following nominating committee for 1937: Arthur H. Smith, chairman, Walter H. Eddy, Lydia J. Roberts, Irvine McQuarrie and T. M. Carpenter.

The group recognized the passing of Prof. Lafayette B. Mendel who had been president at the first scientific session of the American Institute of Nutrition. In his passing the society has lost a revered and loyal member, and a wise and distinguished leader.

Henry C. Sherman moved that a vote of thanks be extended to the local committee of the Institute and that of the Federation of the American Society of Experimental Biology for the splendid arrangements provided for the meeting and for the many courtesies extended to the members and their guests, all of which added so much to the success and enjoyment of the meeting. Motion carried unanimously.

The noon luncheon in the Sun Room of the Hotel Washington was attended by 150 members and guests.

At the subscription dinner at the Garden House of the Dodge Hotel Drs. Eugene F. Dubois and W. C. Rose spoke on the distinguished, inspiring and revered services of Graham Lusk and Lafayette B. Mendel in "The Development of Nutrition in America." The dinner was attended by 116 members and guests and approximately 40 to 50 who were unable to be accommodated for the dinner were present for the talks.

Respectfully submitted,
ICIE G. MACY,
Secretary.

PAST OFFICERS AND MEETINGS

1933 Organization meeting, Cincinnati, Ohio, April 10.
Henry C. Sherman, president; Mary S. Rose, vice-president; John R. Murlin, secretary-treasurer; Eugene F. DuBois and Graham Lusk, trustees.

1934 New York City, March 28 (constitution adopted).
Lafayette B. Mendel, president; Henry C. Sherman, vice-president; John R. Murlin, secretary-treasurer; Mary S. Rose and Eugene F. Dubois, trustees.

1935 Detroit, Michigan, April 10.
John R. Murlin, president; Eugene F. DuBois, vice-president; Icie G. Macy, secretary; William M. Boothby, treasurer; Agnes Fay Morgan, Arthur H. Smith and Roland M. Bethke, councillors.

1936 Washington, D. C., March 25.
John R. Murlin, president; Eugene F. DuBois, vice-president; Icie G. Macy, secretary; George R. Cowgill, treasurer; Arthur H. Smith, Roland M. Bethke and Leonard A. Maynard, councillors.

ABSTRACTS OF PAPERS READ

The availability of the constituents of the green leaf. M. K. Horwitt (by invitation), G. R. Cowgill and L. B. Mendel, Department of Physiological Chemistry, Yale University, New Haven, Connecticut.

Using spinach as an example, a comparison has been made of the constituents of the green leaf which are digested by enzymes in vitro, with the total proximate principles as determined by standard methods of food analysis. Approximately 90 per cent of the nitrogen was made soluble by enzymatic activity, but only 68 per cent of the total amino nitrogen was liberated by digestion with pepsin, trypsin and erepsin, as compared with a 90 per cent liberation of the amino nitrogen of casein by the same technic. Investigation of the character of the insoluble nitrogen revealed that about 5.7 per cent of the total nitrogen is fat-soluble; even more is ammonia and amide nitrogen. Evidently, use of the expression N $\times$ 6.25 cannot give a good estimation of the available protein.

Less than 50 per cent of the fat-soluble fraction of the green leaf was true fat. Similarly, only an appreciable part of the so-called carbohydrate material proved to be of the type which the body can utilize. Studies on the inorganic constituents of the green leaf indicated that only about 30 per cent of the calcium and about 40 per cent of the iron in spinach is rendered soluble by in vitro digestion.

Practical methods have been devised constituting an application of this research to foods in general.

The biological value of heat-treated proteins. W. H. Seegers (by invitation), H. W. Schultz (by invitation) and H. A. Mattill, Biochemical Laboratory, State University of Iowa, Iowa City.

In three series of experiments on twenty-four animals the biological value (Mitchell) of finely ground beef muscle (round) dried at low temperature and fed at a level of 5 per cent protein was not lowered by previous autoclaving for 1 hour at 15 pounds pressure. The heating of casein at 120°C. for 2 hours or at 150° for 30 minutes, as well as various common purification processes likewise had no effect on the biological value of casein measured on two further series of animals. These and earlier observations on heated proteins emphasize the relation of digestibility to biological value, the latter tending to remain unaltered under these conditions if digestibility is not changed.

Further information is also presented on the lowered biological value figures when there is inadequate food consumption, and on the progressive lowering of endogenous urinary nitrogen with prolongation of the nitrogen-free periods, as this affects the determination of biological value. The significance of a relatively high biological value (23 to 54, in five animals) of a tryptophane-deficient diet is discussed.

Replacement values of several proteins in human nutrition. Emma E. Sumner, H. B. Pierce and J. R. Murlin, Department of Vital Economics, University of Rochester, Rochester, N. Y.

Human subjects vary greatly in their ability to ingest a low nitrogen diet, which furnishes 65 to 72 per cent of its total calories from pure carbohydrate food materials. Lack of interest in food of any kind, nausea and even vomiting or diarrhea may result, depending on the individual. If these conditions develop, the subject is definitely in a pathological condition and the results obtained are not trustworthy. In order to insure a more liberal basal diet, thereby avoiding these unpleasant symptoms, the replacement rather than the biological value was determined. Taking egg protein as 100 per cent, the capacity of the proteins of milk, milk and bakers yeast, egg and bakers yeast and wheat endosperm to replace egg protein was studied in eight men and two women subjects at a 4- and a 3-gm. level of nitrogen intake, respectively. Approximately 85 per cent of the total dietary N came from the protein to be studied, the balance from the basal constituents. During the yeast periods 0.8 gm. of egg or milk nitrogen was replaced by an equal amount of yeast N. Calculated on the basis of replacement value the following percentages were obtained: Dried whole milk powder 84, fresh pasteurized milk 81, whole milk powder and yeast 70, fresh milk and yeast 75, egg and yeast 88, wheat endosperm 87.

Is the metabolic nitrogen balance an accurate measure for adequate protein consumption in children? Icie G. Macy, Helen A. Hunscher, Frances Cope Hummel (by invitation), Mary F. Bates (by invitation) and Marsh W. Poole (by invitation), Research Laboratory, Children's Fund of Michigan, Detroit, Michigan.

Nitrogen data accumulated from 375 5-day balances on twenty healthy growing children ranging in age from 4 to 10 years and living within the same delightful and wholesome environmental conditions show enormous unpredicted fluctuations from period to period during runs extending over 60 to 150 continuous days although the dietary nitrogen intake remained constant throughout. Furthermore, with identical food intakes there were marked differences in the degree of nitrogen stored by individual children of similar size and age all with equally healthy satisfactory nutritional state as judged by rigid medical examinations and physical growth records preceding and during the period of observation. The magnitude of observed fluctuations in nitrogen retention of children will be discussed.

The results obtained in this large series of observations indicate that even for long continuous metabolic periods the nitrogen balance method for determining protein requirement of children is an inadequate measure since there are factors other than food that are playing determinate roles in nitrogen metabolism of growth. Subsequent reports will discuss some of these factors.

Dietary standards in childhood. Frederick F. Tisdall, Department of Paediatrics, University of Toronto, and the Hospital for Sick Children, Toronto.

Studies now in progress on humans indicate that further investigations should be made on our present dietary standards in childhood, with a view to their revision.

Our so-called 'good diet' may not contain the optimal amount of the vitamin B-complex. The amount of vitamin D required during infancy is less than usually prescribed. The iron requirements for the prevention and cure of nutritional anemia in infancy and childhood should be considered in terms of available iron rather than total iron. No evidence has been found of a lack of vitamin A in the average good diet. A satisfactory method for the estimation of the requirements for vitamin C has not been found.

The basal metabolism and heat of vaporization of premature infants. Harry H. Gordon (by invitation) and S. Z. Levine, New York Hospital and Department of Pediatrics, Cornell University Medical College, New York.

The basal metabolism of twenty-two premature infants of 1 to 70 days and 1200 to 3380 gm. was determined by indirect calorimetry in sixty-four observations. Simultaneous observations of the elimination of water through skin and lungs were made. Alcohol checks at a level of heat production comparable to that of the infants (4 to 10 calories per hour) controlled the reliability of observations.

The basal metabolic rate of premature infants in calories per kilogram (average of 58 in these observations) closely approximates that of full term infants. The lower rate of premature infants in calories per square meter may represent an intrinsic abnormality or may be explained by their relatively larger surface area. The percentage of heat production dissipated in vaporization of water averaged 29 (24 to 33 per cent) as compared with 26 per cent (23 to 30 per cent) for full term infants.

A defective heat production or a disturbance in the mechanism of heat loss in vaporization cannot be postulated with certainty as a cause of hypothermia in premature infants without further investigations of heat loss and heat production under taxing environmental conditions.

The average respiratory quotient of 0.88 obtained as early as the first week of life indicates a facile oxidation of lactose. The urinary nitrogen of these subjects, fed breast milk, represented only 4 per cent of the total calories produced.

Parallel determinations of human alveolar carbon dioxide and respiratory quotient after ingestion of galactose. Thorne M. Carpenter, Nutrition Laboratory of the Carnegie Institution of Washington, Boston.

In order to determine whether the marked change in the respiratory quotient after ingestion of galactose was due to combustion only, experiments were made with a human subject in which determinations of the respiratory quotient and of the alveolar carbon dioxide were carried out in 15-minute periods during a base-line hour and for 3 hours after the ingestion of the sugar. Controls with 250 cc. water showed no change in either respiratory quotient or alveolar carbon dioxide

during 4 hours. With 25 and 50 gm. of galactose, there was a fall in the alveolar carbon dioxide. The fall with 50 gm. was marked and lasted for over 2 hours. When 25 gm. each of galactose and of glucose were given together there was a slight fall in the alveolar carbon dioxide. Fifty grams of lactose showed no definite change in the alveolar carbon dioxide. The experiments indicate that at least part of the rise in the respiratory quotient after the ingestion of galactose can be ascribed to a change in the alveolar carbon dioxide, and, therefore, all of the rise in the respiratory quotient is not due to an increased combustion of the sugar. It is concluded that there is a formation of organic acids in the intermediary metabolism of galactose.

Influence of a diet deficient in salts on the total energy metabolism of rats. Max Kriss and Arthur H. Smith, Department of Physiological Chemistry, Yale University, New Haven, Connecticut.

Young rats fed a diet poor in inorganic salts for 3 months showed the following differences in the total respiratory metabolism, as compared with their calorie-controls, whose diet contained 4 per cent of Osborne and Mendel salt mixture.

After feeding at supermaintenance or maintenance levels, the average hourly carbon dioxide production during 8-hour periods, and the calculated heat elimination, uninfluenced by effects of activity, were markedly higher for the low-salt animals than for the controls, although the body weights of the former were less. This effect appeared at the end of the first month and persisted during the last 2 months.

The respiratory quotients were significantly lower with the low-salt animals than with the controls during the last 2 months. The mineral deficiency resulted in the oxidation of relatively larger proportions of fat and smaller proportions of carbohydrate, while it was practically without effect on the proportions of protein metabolized.

The low-salt animals eliminated smaller proportions of heat through vaporization of water than did the controls.

In spite of the noted differences in the total heat production, there were no significant differences in the specific dynamic effects of the diets compared, as these effects are generally defined.

Prevention of fluorine toxicosis in the rat with aluminum chloride. George R. Sharpless (introduced by Icie G. Macy), Department of Laboratories, Henry Ford Hospital, Detroit.

Rats which develop white and defective incisors on a diet containing 250 parts per million of sodium fluoride show normal incisors, except for fine transverse pigment lines visible under a hand lens, when aluminum chloride is added to the diet. Fifty-six thousandths per cent aluminum in the diet protects for a short time, and 0.224 per cent aluminum protects more than 6 months (the observed period). With a diet containing 1000 parts per million of sodium fluoride (an amount that restricts body growth) and 0.224 per cent aluminum as the chloride, growth is normal, but the incisors show broad yellow and white bands, and the uppers may overgrow. If 2 per cent calcium carbonate is added to this diet, the protection is more complete.

If the diet containing aluminum chloride is made chemically alkaline, the protection is not as great. If the acidity is increased, the aluminum gives no greater protection.

Salts of other substances which form insoluble fluorides or complex ions with fluorine such as lanthanum, cerium, thorium and boron, were tried. They were found to have either no effect or, as in the case of lanthanum, slight protective action.

It is suggested that, since aluminum is not absorbed by the rat, the protection must take place in the intestinal tract, probably by the formation of a slightly dissociated compound of aluminum and fluorine.

Relation between dose level of vitamin A and the animal response. Walter H. Eddy, Laboratory of Physiological Chemistry, Teachers College, Columbia University, New York City.

Analysis given of the behavior of 700 rats in tests conducted by the Pharmacopeia method using U. S. Reference oil and seventeen each of commercial cod and halibut liver oils as sources of vitamin A.

The outstanding feature of the analysis is the wide variation in rat response to any given dose level in spite of compliance with the requirements of the U.S.P. method in basal diets, selection of groups, sex distribution, etc.

Specific study was made to determine whether this variation could be attributed to variation in rats at weaning age, variation in weight at end of clearing period, degree of depletion at beginning of assay period due to definition of 'declining weight,' method of calculating the 28-day gain, seasonal variation in response, distribution of sexes in groups.

While some of these factors have influence, it was found in this series that variation begins during the clearing period and that the condition of the rat when put on test is probably a much more significant determinant of his behavior during the assay period than his sex, his weight at beginning of assay, or the degree of decline in weight just before receiving the assay source of vitamin A.

The analysis suggests the necessity of not only a reference source of vitamin A but a reference curve for each assay laboratory to arrive at truly significant comparisons between unknowns and reference samples, a reference curve by which probable error is reduced to minimum by the behavior of at least fifty rats on each reference dose level.

It also suggests that spectrometric data may find practical use in reducing the number of exploratory levels necessary to arrive at bio-assay results of real significance.

The effect of single administrations of hepatoflavin on the appetite and body weight of rats on a flavin-deficient diet. Wendell H. Griffith, Department of Biochemistry, St. Louis University School of Medicine, St. Louis.

Young rats on a flavin-deficient diet survive for many weeks although growth failure is complete. During this survival period there is a striking response to the oral or parenteral administration of hepatoflavin. Single administrations of this vitamin produce growth responses proportional to the amount of flavin used. Rats which are stationary in weight between 50 and 70 gm. may increase in

weight as much as 10 gm. in the 24-hour period following the administration of the supplement. With smaller amounts of flavin the weight decreases after the first day and returns to the original level. With larger amounts growth may continue for several days although the maximum effect occurs during the first 24-hour period. The administration of the vitamin stimulates the appetite so that the consumption of food and of water is immediately increased. It appears probable that the greater part of the body weight increment in these experiments resulted from increased water intake.

The growth response due to graded amounts of autoclaved liver, aqueous liver extract and hepatoflavin has been determined by this single administration procedure. Negative results were obtained in control experiments in which extra vitamin B was used as the supplement. In all cases groups of eight to twenty rats were used for each test. This method of detecting flavin is of advantage because it is subject to quantitative interpretation, because it gives qualitative results within 24 hours, and because the test rats may be used repeatedly.

Concerning two types of rat dermatitis. Albert G. Hogan and Luther R. Richardson (by invitation), Laboratory of Agricultural Chemistry, University of Missouri, Columbia.

If the basal diet is supplemented with tikitiki as a source of the B complex the rats become denuded, with relatively mild skin lesions, and finally succumb. These symptoms are described by Goldberger, by Sherman, and by others, and they are healed by flavine. If tikitiki is replaced by an irradiated water extract of yeast, the rats develop a severe dermatitis which is healed by tikitiki and by wheat germ oil. If rats that have developed dermatitis are given crystalline vitamin B plus wheat germ oil they heal promptly but fail to grow. If they are given the same vitamin B plus flavine they continue to decline. If rats that have become denuded are given vitamin B plus flavine they heal but gains in weight soon cease. If they receive vitamin B plus wheat germ oil the decline continues. Either type of lesion is healed by crystalline vitamin B plus wheat germ oil plus flavine but the animals only attain one-half the normal mature weights. This mixture contained more of the known vitamins than did a supplement of tikitiki plus flavine, but the latter combination permitted the rats to attain normal size. Taken collectively the evidence demonstrates that the B complex contains three factors and indicates that it contains at least four.

The multiple nature of vitamin D. II. The vitamins D of fish oils. Charles E. Bills, O. N. Massengale (by invitation), Miriam Imboden (by invitation) and Helen Hall (by invitation), Research Laboratory, Mead Johnson and Company, Evansville, Indiana.

By means of a newly developed precision technic for the assay of vitamin D with chickens, the liver oils of twenty-five species of fish were compared with cod liver oil, irradiated ergosterol and irradiated 7-dehydrocholesterol. Rat unit for rat unit, the oils showed large differences in effectiveness for chickens. By the elimination of alternative explanations (influence of vitamin A, presence of free acid, and existence of vitamin D in conjugated forms), it was concluded that the differences indicate the existence of more than one form of vitamin D in the oils.

The liver oil of the albacore, Thunnus germo, had the lowest relative efficacy, about 0.12 that of cod liver oil; and the liver oil of the white sea bass, Cynoscion nobilis, had the highest relative efficacy, 2.6 times that of cod liver oil. This extreme difference of about twenty times is much greater than the probable errors of assay. No fish oil had as low an efficacy ratio as irradiated ergosterol. Cod liver oil showed about the same effectiveness, rat unit for rat unit, as irradiated 7-dehydrocholesterol.

Oils of related species, particularly the several tunas, differed widely. Our findings support the contention of Jordan and Evermann ('26) that the big tunas formerly classed as Thunnus thynnus actually comprise several species, of which we have studied T. saliens, T. orientalis and T. secundodorsalis.

The antirachitic actions of irradiated sterols and derivatives thereof. Elizabeth M. Koch (by invitation) and F. C. Koch, Department of Biochemistry, Chicago.

It has been demonstrated that spinal cord cholesterol, as prepared by The Wilson Laboratories, which shows the typical absorption bands of ergosterol, is many times as effective, on a rat unit basis, as ergosterol for the prevention of leg weakness in chicks.[1] This suggests strongly that the contaminant in spinal cord cholesterol is not ergosterol. 7-dehydrocholesterol was prepared as described by Windaus, Lettré and Schenck.[2] Its spectrum when calculated on a molecular basis, we found to be identical with that of ergosterol. Its potency for avian rickets as determined by the chick test, is at least thirty times that of an equivalent number of rat units of viosterol. This is presented as evidence that the contaminant in the spinal cord cholesterol is 7-dehydrocholesterol rather than ergosterol. The typical ergosterol bands cannot, however, be detected in purified, heated cholesterol which on irradiation is equivalent to spinal cord cholesterol both for rats and for chicks. This cholesterol was recrystallized after heating from ethyl acetate and alcohol until there was no longer general absorption in the region of the ergosterol bands. These observations are given as evidence that the provitamin D of heated cholesterol is still another substance than 7-dehydrocholesterol.

Corn oil phytosterol, which shows the typical ergosterol bands, is about three times as potent, measured by the chick test, as an equivalent number of rat units of viosterol. This indicates that the provitamin D corn oil phytosterol is still a fourth substance.

All these products, with the exception of viosterol, were irradiated in the dry crystalline state. It is possible that a more effective technic of irradiation may change these relationships.

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[1] Koch, Elizabeth M. and F. C. Koch 1935 Fractionation studies on provitamin D. Science, vol. 82, pp. 394–395.

[2] Windaus, A., H. Lettré and Fr. Schenck 1935 Über das 7-Dehydrocholesterin. Annalen, Bd. 520, S. 98–106.

Factors influencing the vitamin C content of vegetables. Donald K. Tressler (by invitation) and Guilford L. Mack (by invitation), New York State Agricultural Experiment Station, Geneva, New York, and C. G. King, Chemistry Department, University of Pittsburgh, Pittsburgh, Pennsylvania.

A detailed study of the ascorbic acid content of vegetables has been begun. The influence of variety, maturity and freshness of the vegetable and the effect of growing conditions, soil and fertilizer on the vitamin C content are being investigated. The effect of cooking and various methods of preservation on the ascorbic acid content of these vegetables are also being studied.

Variety has been found to be an important factor in the case of certain vegetables, e.g., peas and snap beans; in rhubarb, spinach, cabbage and some others it is of little importance. Most vegetables lose their vitamin C rapidly if held at room temperature after harvesting. Refrigeration greatly reduces the rate of loss. The percentage of ascorbic acid in peas decreases as they mature, whereas there is little difference in spinach, snap beans, and rhubarb at different stages of maturity.

Preliminary cooking studies indicate that a considerable proportion of the ascorbic acid passes into the water during cooking by boiling, but the total amount of ascorbic acid decreases but little.

Blanching sufficient to inactivate catalase is necessary in preparing vegetables for freezing in order to retain the ascorbic acid content of frozen vegetables at the usual cold storage temperatures. Thawed vegetables slowly lose vitamin C.

Preliminary work has shown that both soil and growing conditions have a marked effect on the ascorbic acid content of spinach.

ABSTRACTS OF PAPERS READ AT THE SYMPOSIUM ON NUTRITIONAL HISTORY OF THE PRESENT DAY DEPRESSION

Nutrition in emergency relief. Adelaide Spohn, Elizabeth McCormick Memorial Fund, Chicago, Illinois.

The avalanche of unemployment which started in 1929 brought to local, state and federal agencies the problem of providing for large masses of the population that previously had been self-supporting. For economic as well as humanitarian reasons the conservation of the health of individuals on relief was the primary concern of relief administrations. Food was therefore considered the most important item in the family budget and received first consideration. Many states adopted the standards outline by Stiebeling and Ward in 'Adequate Diets at Minimum Cost' (U. S. D. A. cir. 296). The food allowances are based on individual needs of age, sex and activity and are expressed in terms of general food groups which are readily modified to take advantage of local food resources and conform with individual food preferences. The desire for economy and the fear that liberal relief standards would encourage dependency combined to keep relief standards below adequacy in some states and in many local communities. In July 1935 the average relief benefits for the country reached a peak at $29.64 per family per month, individual states ranging from $8.75 to $49.08. The variation in these figures are accounted for by a complexity of factors but doubtless the most important of these is actual difference in standards of relief.

Studies concerning the nutritive value of foods purchased by low-income and relief families show that adequate allowances for food do not always assure an adequate diet. Such studies generally show a greatly diminished use of milk, vegetables, fruits and cereals, and an excess of sugar and fats, resulting frequently in diets deficient in calcium, phosphorus, iron and vitamins.

Nutritional history of the present day depression. James S. McLester, Professor of Medicine, University of Alabama, President; American Medical Association.

Studies of the effects of the present day depression upon the nutritive state of the American school child are somewhat conflicting, but the preponderance of evidence is that on the whole the child has not suffered. The median weights of children of the same age groups among 20,000 pupils of the Birmingham public schools were essentially the same in 1934 as in 1927. Twenty-five selected physicians, living in different parts of the country, report that, on the whole, they see no more malnutrition now than before the depression.

ABSTRACTS OF PAPERS READ BY TITLE

Effects of increased metabolism on the ketone body excretion of the depancreatized dog. S. B. Barker (introduced by William H. Chambers), Department of Physiology, Cornell University Medical College, New York City.

Exercise, injection of dinitrophenol, or administration of desiccated thyroid substance was used as a means of increasing the metabolism of depancreatized dogs. The total ketone body production was obtained by measuring the acetone excreted through the lungs as well as by the usual urine analysis. Determination of the respiratory exchange and of glucose and nitrogen excretion indicated that the extra heat was derived from fat rather than from carbohydrate.

Exercise which doubled the fat metabolism increased commensurately the total ketone body output in only four of fifteen experiments. Acetone in the breath always increased, up to fourfold, but, at the same time, excretion of acetone bodies by the kidneys was diminished. During the recovery period the air acetone returned to the basal level, whereas there was no regularity about the amount of ketones in the urine.

When the heat production was increased for several hours by small doses of dinitrophenol, a fall in total ketone body excretion occurred despite an increased amount of acetone in the expired air.

The maintenance of an increased basal metabolism for several days by administration of desiccated thyroid was accompanied by a low ketone body elimination.

In these experiments there was no correlation between the increased fat metabolism and changes in acetone body excretion.

A comparison of intraperitoneal injection versus oral administration of iron upon blood regeneration in nutritional anemia of the rat. Howard H. Beard and Thomas S. Boggess (by invitation), Department of Biochemistry, Louisiana State University, New Orleans.

Young rats were made anemic by feeding on cow's milk containing an average of 0.11 mg. Cu per liter as determined by the diethyl dithiocarbamate method. They were then fed or injected with different amounts of a colloidal Cu-free ferrous and ferric hydroxide preparation (Eisen Diasporal) until blood regeneration was complete. The total amount of Fe required under these conditions was then compared. From five to eight anemic animals were used for each dose of the elements.

Two milligrams colloidal Fe injected weekly for 4 weeks (8 mg.) was as effective on blood regeneration as 2 mg. Fe ingested daily by mouth for 3 weeks (42 mg.). Twenty-six hundredths milligram Fe injected every other day for 3 weeks (2.73 mg.) was as effective as the ingestion of 0.26 mg. daily for 5.5 weeks (10 mg.). The injection of optimum doses of Cu or Mn with Fe gave no better effect on blood regeneration than the injection of the same dose of Fe alone.

Blood regeneration in nutritional anemia of the rat occurs when only 19 to 27 per cent of pure Fe is in the body as compared to that in the intestinal tract.

The significant relation between growth and appearance of cataract in rats given graded amounts of vitamin G. Paul L. Day and William J. Darby (by invitation), Department of Physiological Chemistry, School of Medicine, University of Arkansas, Little Rock.

More than 200 young rats, weighing between 30 and 45 gm., were used in a series of vitamin G assays. For the first 2 weeks they were given a deficient diet only, after which time they were given daily supplements of vitamin-containing foods. Control animals receiving no supplement were kept until death, whereas the animals receiving supplements were killed after 10 weeks.

Eighty-six per cent of seventy-eight controls developed cataract before death, although only 63 per cent showed such eye changes during the 10-week period. The rats receiving vitamin supplement, grouped according to growth during the 10-week period, showed the following incidence of cataract: Animals growing 20 gm. or less—39 per cent cataract; animals gaining from 21 to 40 gm.—26 per cent cataract; animals gaining from 41 to 60 gm.—14 per cent cataract; animals gaining more than 60 gm.—0 per cent cataract. It is thus apparent that there was an inverse relationship between growth and appearance of cataract; that is, the greater the growth, the lower the incidence of cataract. This would seem to indicate that, under the conditions of the experiment, growth was a measure of the cataract-preventive property of the supplement. These data also indicate that only a small amount of the vitamin is required to prevent the appearance of cataract.

Further studies on vitamin B_4. O. L. Kline (by invitation), C. A. Elvehjem and E. B. Hart, Department of Agricultural Chemistry, University of Wisconsin, Madison.

The synthetic ration for the production of vitamin B_4 deficiency in chicks which was described previously by Keenan, Kline, Elvehjem and Hart[1] has been improved to give more consistent results. Reprecipitated casein has replaced the crude casein, the yeast reduced to 2 per cent of a yeast high in vitamin B_1 the liver residue decreased to 2 per cent, and 2 per cent of crude liver extract added. When these changes were made acute vitamin B_4 deficiency appeared quite consistently, but the results were complicated by the occurrence of severe lesions in the lining of the gizzards. The addition of 5 per cent of water extracted lung tissue supplied sufficient amounts of the gizzard factor to prevent this deficiency. The final ration (ration 452) has the following composition: Dextrin 64, purified casein 18, salts I 5, brewer's yeast 2, autoclaved liver residue 2, crude liver extract 2, cod liver oil 2. Chicks hatched from eggs produced by hens on a uniform ration showed acute symptoms of vitamin B_4 deficiency after 2 to 4 weeks on this ration. Chicks from commercial hatcheries do not develop the symptoms as uniformly and often require 5 to 6 weeks before typical symptoms appear. The addition of 15 per cent of peanuts, brain tissue, or kidney gave complete protection against paralysis and produced normal chicks. Concentrated preparations of the vitamin from peanuts are now being studied.

[1] Keenan, J. A., O. L. Kline, C. A. Elvehjem and E. B. Hart 1933 J. Biol. Chem., vol. 103, p. 671.

The effect of cortin on respiratory metabolism in erect posture. Fred A. Hitchcock and George W. Thorn (by invitation), Laboratories of Physiology, Ohio State University, Columbus.

A study has been made by means of the Tissot-Haldane technic of the oxygen consumption of a group of men and women before, during, and after the administration of cortin. The oxygen consumption and respiratory quotients were determined 1) while the subjects were in a basal state and 2) while they were standing erect. No significant change in the basal oxygen consumption was observed. In all but one of the subjects, however, the administration of cortin was accompanied by a pronounced drop in the oxygen consumed in maintaining the erect posture. It appears therefore that the increase in oxygen consumption over and above the basal requirement necessary in standing erect is lessened by cortin. In some cases the cortin seemed to produce a rise in the respiratory quotient but this effect was not consistent and so is probably not significant.

The effect of calcium on the iron assimilation of the rat foetus. Seymour W. Kletzien and Clara Kingdon (by invitation), State Institute for the Study of Malignant Disease, Buffalo, New York.

In a previous report it was shown that the iron assimilation of the growing rat was retarded when calcium, strontium, magnesium, beryllium or barium were added to an otherwise complete diet in an amount equivalent to 1 per cent of calcium carbonate. Because the mammalian foetus with one known exception is normally born with a reserve of iron to tide it over the suckling period when the iron intake is scanty, it seemed desirable to determine what effect if any a calcium enriched diet would have on the iron assimilation of the pregnant rat and its young.

The consumption of a complex stock diet supplemented with additional calcium by female rats having a reproductive history uncomplicated by lactation and averaging six litters showed a progressive decrease in the average iron content of each succeeding litter in contrast to the controls, whose litters maintained a higher and a more constant iron content throughout. Likewise, the experimental females revealed a lower average iron content of the carcass, blood, liver and spleen and a lower hemoglobin index than the controls when sacrificed at the termination of the experiment. With a more limited iron intake the depressing effect of the calcium additions was accentuated. These experiments it is believed, indicate a fundamental difference in the nutritional needs of pregnancy and those of lactation.

Utilization of hexoses by excised rat tissues. M. Elizabeth Marsh, Department of Vital Economics, University of Rochester, Rochester, N. Y.

Determination of O_2 consumption and R.Q.'s were made on tissues from fed rats and rats fasted 24 to 48 hours, in Ringer-phosphate solutions with and without sugars.

With kidney cortex slices the increase in O_2 uptake with glucose averaged 28 per cent for the fed and 19 per cent for the fasted groups. The cause of the increase is uncertain, however, since the R.Q. did not change significantly in either group. Fructose raised the O_2 consumption still higher, 50 per cent and 35 per cent, respectively, and the rise in R.Q. agreed, in both groups, within 0.003 of theoretical calculated on the assumption that all the extra O_2 was occasioned by the complete combustion of the sugar.

Liver tissue made little or no use of added glucose. In the fed animals the O_2 was usually slightly depressed by the glucose and the R.Q.'s for both groups averaged the same with and without the glucose. Fructose raised both the O_2 and the R.Q. as the kidney, but to a lesser degree and with two other differences noted: 1) the O_2 increase was greater in the fasted than in the fed group (18 per cent and 7 per cent, respectively) and 2) the average R.Q. was, in both groups, 0.063 higher than the theoretical R.Q. Is the liver converting a portion of the fructose directly to fat?

Galactose was without effect upon the O_2 and upon the R.Q. with both kidney and liver.

The relation of vitamin B to fat deposition in the liver. E. W. McHenry, Department of Physiological Hygiene, School of Hygiene, University of Toronto, Toronto, Canada.

Young male or female rats fed on a diet low in choline but otherwise adequate, develop markedly fatty livers. This diet provided 5 gamma of crystalline vitamin B per rat per day. Rats fed a similar diet, except that vitamin B is absent, have about one-half as much liver fat. This effect could not be observed when choline was contained in the diet since that substance effects a reduction in liver fat. Greater amounts of vitamin B are required to secure fatty livers in adult rats. A study has been made of the time required to produce this effect. From the many possible explanations of this action of vitamin B, two have been selected as more probable. The increased liver fat might result from 1) increased absorption of fat from the intestine, or 2) it might be due to an effect of vitamin B, directly or indirectly, upon the transport of fat to the liver. Measurements of intake and excretion of fat indicate that the first explanation is unlikely and this is in agreement with the results of other workers, notably H. M. Evans. Since several investigators have shown that a deficiency of vitamin B affects the pituitary it is considered more likely that the production of fatty livers caused by feeding the vitamin in the absence of choline is related to the action of 'ketogenic' hormone which has been shown to cause an increased deposition of fat in the liver. Studies on hypophysectomized rats are in progress.

The effect of depancreatization and ligation of the pancreatic ducts on the liver lipids of dogs. Elaine P. Ralli and Clara H. Present (by invitation), The Nutrition Laboratories of the Department of Medicine, New York University College of Medicine, New York City.

Six dogs, kept on a basic diet, to which was added either cod liver oil, hydrogenated vegetable oil, or carotene and corn oil, were studied as follows. After a control period of at least 6 weeks on the diet, a portion of the liver was removed and the animal was depancreatized or the pancreatic ducts were completely ligated. The liver was analyzed for total lipid, unsaponifiable material, total fatty acids, iodine number of the fatty acids, lipid phosphorus, and total cholesterol, free cholesterol and cholesterol esters. After a period, varying from 3 to 8 months, during which time, the dog received the same diet as during the control period, and if diabetic was controlled by insulin, the animal was sacrificed and the liver again analyzed as before. The results showed that in both the ligated and depancreatized dogs, the total lipid and total fatty acids were greatly increased above the normal, and that the increase was for the most part in neutral fat. The iodine number fell in all cases. The total cholesterol was increased, due to an increase in cholesterol esters. The lipid phosphorus was decreased. The total amount of unsaponifiable increased in some animals, decreased in others. Three dogs received 10 gm. of crude egg yolk lecithin daily. It apparently had no effect in preventing the deposition of fat in the liver of the depancreatized or ligated animal.

Further studies on the relation of diet to goiter. Roe E. Remington, E. J. Coulson (by invitation) and Harold Levine, Department of Nutrition, Medical College of the State of South Carolina, Charleston, South Carolina.

A study of 125 experimental groups, comprising 1021 rats on diet with varying degrees of iodine deficiency reveals that definite correlations exist between thyroid size and iodine content and between thyroid size and dry matter content, and that curves can be drawn expressing these relationships. It is pointed out that the critical part of the curve is within a range of iodine content in the dried gland below 0.08 per cent, and that experiments on low iodine goiter must be done within this range.

Results on feeding dried milk, dried oysters and dried haddock indicate that the goiter-preventing value of these foods in proportional to their iodine content and of the same order of magnitude as that of potassium iodide. Irish moss gave a less favorable result.

The relation of the vitamin A content of the poultry ration to egg production, mortality and hatchability. Walter C. Russell, M. W. Taylor (by invitation) and D. F. Chichester (by invitation), Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station and Rutgers University, New Brunswick, New Jersey.

In a study of the causes of high mortality in laying flocks, vitamin A was determined by the antimony trichloride method in the livers of 154 pullets and hens submitted from farm flocks for autopsy. A large percentage of these birds were diseased, but the vitamin A values of their livers were not essentially different from those of sixty-nine birds considered to be normal.

Records of seven pens of pullets (forty-eight to the pen), whose vitamin A varied from 880 to 66,000 U.S.P. × 1934 units per kilogram of feed consumed, during a 12-month period, revealed that the largest quantity of the factor ingested did not increase production, decreased mortality only slightly, and did not improve hatchability, as compared with the smallest quantity fed. The vitamin A values for the livers of the birds which died fell in essentially the same order as the vitamin A contents of rations consumed by the seven pens, and the amount of the factor in the eggs of the lowest vitamin A group was markedly less than from the eggs of the highest group.

The results of both lines of the investigation led to the conclusion that high mortality in poultry flocks is not due to a vitamin A deficiency in the usual poultry ration.

Role of the hypophysis in the dextrose tolerance curve. Samuel Soskin, The Metabolic Laboratory of the Department of Physiology, Michael Reese Hospital, and the Department of Physiology, The University of Chicago.

At the previous meeting of this society it was shown that the liver and not the pancreas is essential to the normal dextrose tolerance curve. It was concluded that our results indicated the existence of a homeostatic mechanism of the liver, whereby the organ decreases its supply of sugar to the blood in response to an influx of exogenous sugar. In this mechanism, the rise in blood sugar

consequent to sugar administration may be regarded as the stimulus which activates the hepatic regulating mechanism, while the insulin in the circulating blood is merely one of the factors which determines the blood sugar level or threshold at which the homeostatic reaction comes into play.

In th ent report it is shown that the hypophysectomized dog yields dextrose tolerance curves of perfectly normal proportions, but transposed to subnormal blood sugar levels. On the other hand, the hypophysectomized-depancreatized animal maintained without insulin also yields normal dextrose tolerance curves, but only at high blood sugar levels. Thus the hypophysis, like the pancreas, is a factor in determining the threshold of hepatic blood sugar regulation, but is not essential to the mechanism itself.

These results, and other data, yield a rational interpretation of the older clinical observations on the metabolic disturbances in conditions of hypo- and hyperactivity of the hypophysis, and of the newer experimental observations on the modified diabetic manifestations of the 'Houssay' animal.

Factors necessary for successful reproduction and lactation in the rat. Pearl P. Swanson and Elizabeth Dyar (by invitation), The Nutrition Laboratory of the Foods and Nutrition Department, Iowa State College, Ames.

A complete breakdown of the reproductive performance has been observed on a diet supposedly adequate in all known essentials and unlike, in composition, any food mixture as yet reported. The ration was composed of dried canned pork muscle, cornstarch, agar agar, butterfat, salts, cod liver oil and yeast. Assay showed that the diet carried adequate vitamin E. One hundred rats were studied. The rate of growth was depressed, especially in later generations. The average life span was short.

The oestrous cycles were normal during the first 3 months of breeding. After that, they lengthened and the smear showed cornified cells only. Sexual activity ceased. In the first generation, the rats were 100 per cent sterile.

Embryonic development was normal. Positive matings were followed by successful implantations. Resorptions did not occur but at parturition, at the end of a somewhat lengthened gestation period, one-third of the rats died. At birth, many of the rats were dead or malformed. Fifty-five per cent of the living young died during the first week after birth. Young that survived this period grew at nearly a normal rate.

The supplementary values of certain amino acids, linoleic acid, yeast, hydrolyzed yeast, liver and liver concentrates are being tested.